Exopolysaccharide production by Antarctic marine bacteria

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Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any tertiary institution. To the best of my knowledge this thesis does not contain material written or published by another person, except where due reference is made.

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Contents

List of Tables

List of Figures

Abbreviations

The following abbreviations and acronyms have been used in this thesis:

16S rDNA	16S ribosomal RNA gene
Ara	arabinose
CAM	author's Collection of Antarctic Microorganisms
CFB	*Cytophaga-Flexibacter-Bacteriodes*
Da	Daltons
EPS	exopolysaccharides
FAME	fatty acid methyl ester
FID	flame ionization detector
FT-IR	Fourier transform-infrared spectroscopy
Fuc	fucose
Gal	galactose
Gal A	galacturonic acid
Gal N Ac	N-acetyl galactosamine
GC	gas chromatograph
GC/MS	gas chromatograph-mass spectrometer
Glc	glucose
Glc A	glucuronic acid
Glc N Ac	N-acetyl glucosamine
IFREMER	Institut Français de Recherche pour l'Exploitation de la Mer
MA	marine agar
MA+Glu	marine agar amended with 3% glucose (w/v)
Man	mannose

MB+Glu	marine broth amended with 3% glucose (w/v)
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
PHYLIP	phylogeny inference package
Rha	rhamnose
SNB	seawater nutrient broth
TEP	transparent exopolymer particles

Abstract

Antarctic marine bacteria isolated from sea ice and Southern Ocean particulate material were screened for exopolysaccharide (EPS) production. Ten strains were characterized using phenotypic (morphology), chemotaxonomic (whole cell fatty acid profiles) and phylogenetic (16S rDNA sequencing) techniques. These isolates were representatives of four genera including *Pseudoalteromonas, Shewanella, Polaribacter* and *Flavobacterium*, with one strain constituting a new bacterial genus in the family *Flavobacteriaceae*. After further phenotypic characterisation, this strain was given the name *Olleya marilimosa*, gen. nov., sp. nov..

The ten strains were grown in batch culture and the EPS extracted, purified and partially characterized. Crude chemical, monosaccharide and molecular weight determinations showed that the EPS were diverse, even among closely related isolates. All EPS contained uronic acids to varying degrees and some also contained sulfate groups. Two EPS showed the presence of acetyl groups, with pyruvate present in at least one polysaccharide.

The bacteria belong to phylogenetic groups that are dominant in sea ice and Southern Ocean particulate material according to previous studies that used culture dependent and independent techniques. These isolates were psychrotolerant, grew between 2 to 25°C and had growth optima at approximately 20°C. Growth and EPS production of one isolate belonging to the genus *Pseudoalteromonas* was examined at three temperatures: -2°C, 10°C and 20°C. EPS yield at –2°C and 10°C was thirty-fold higher than at 20°C. The EPS showed higher levels of uronic acids at lower temperature.

The metal binding ability of a high molecular weight, highly viscous EPS produced by one sea ice bacterium was examined. High affinities for cadmium and copper were observed at the low concentration of EPS used. These results are a first step in assessing the ability of EPS produced by Antarctic marine bacteria to chelate dissolved trace metal such as iron, which are essential for growth and are limiting primary production in the Southern Ocean.

The examination of EPS production by Antarctic marine bacteria provides new evidence that these biopolymers are abundant and diverse. Partial structural elucidation reveals important structure-function relationships. EPS such as those examined in this study may have a cryoprotective role or may impact on the availability of important trace metals. These findings point to the wider ecological role of EPS within the Antarctic marine environment. This study also provides incentive for further investigation into commercial usefulness of these biopolymers.

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Publications produced from this thesis

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Mancuso Nichols CA, Guezennec J, Bowman JP (2005) Bacterial exopolysaccharides from extreme marine environments, with special with special consideration of the Southern Ocean, sea ice and hydrothermal vents. *Marine Biotechnol* 7(4): 253-271.

Mancuso Nichols CA, Bowman JP and Guezennec J (2005) The effects of temperature on the growth and production of exopolysaccharides by two Antarctic sea ice isolates grown in batch cultures. *Appl Environ Microbiol* 71(7):3519-3523.

Mancuso Nichols CA, Bowman JP and Guezennec J (2005) *Olleya marilimosa* gen. nov., sp. nov., an exopolysaccharide producing marine bacterium from the family *Flavobacteriaceae* isolated from the Southern Ocean. *Int J Syst Evol Microb* 55:1557-1561.

Bowman, JP, Abell, GCJ and Mancuso Nichols, CA (2005) Psychrophillic extremophiles from Antarctica: biodiversity and biotechnological potential. *Ocean and PolarResearch.* 27(2): 221-230.

In a Non-Peer Reviewed Publication:

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*In Conference Publications (*presenting author)*:

Mancuso Nichols CA*, Guezennec J, Nichols PD, Bowman JP Cool Prospects: Exopolysaccharides in Antarctic Bacteria, European Society for Marine Biotechnology IXth Meeting, May 12-14, 2002, Nantes, France, Abstracts P-04.

Mancuso Nichols CA*, Garon S, Bowman JP, Gibson JAE, Nichols PD, and Guezennec J Exopolysaccharide Production by Antarctic Bacteria: Implications for Nutrient Cycling, 104th General Meeting of the American Society for Microbiology in New Orleans, Louisiana, USA, May 23-27, 2004, Abstract number N-204.

Mancuso Nichols CA, Garon S, Bowman JP, Gibson JAE, Nichols PD* and Guezennec, J Exopolysaccharide Production by Antarctic Bacteria: Implications for Nutrient Cycling, Australian Marine Science Association Annual Meeting, Hobart, Tasmania July 6-9, 2004.

Mancuso Nichols CA*, Bowman JP and Guezennec J Cold stimulated production of a novel exopolysaccharide by an Antarctic sea ice bacterium, Australian Society for Microbiology, Sydney, Australia, September 2004. Abstract number PP29.

Mancuso Nichols CA*, Garon S, Bowman JP Gibson JAE, Nichols PD and Guezennec, J Exopolysaccharide Production by Antarctic Bacteria:

Implications for Nutrient Cycling, Australian Society for Microbiology, Sydney, Australia, September 2004, Abstract number P11.09.

Bowman J*, Mancuso Nichols CA, Smith M, Abell G Exploring Antarctic waters: From ACAM to biodiscovery, Australian Society for Microbiology, Sydney, Australia, September 2004, Abstract number SY15.3.

Mancuso Nichols CA*, Garon S, Bowman JP, Gibson JAE, Nichols PD and Guezennec J Exopolysaccharide Production by Antarctic Bacteria: Implications for Nutrient Cycling, Australian Society for Microbiology, Sydney, Australia, September 2004, Abstract number P11.

Chapter 1. Overview

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1.1. Background

In natural systems, microorganisms secrete exopolymers as part of their growth strategy to facilitate adhesion to and colonization of surfaces (Decho 1990, Marshall 1985). Exopolymers provide a medium through which specific biochemical interactions between the bacteria and surrounding cells and tissues may occur (Logan and Hunt 1987). They also protect the microbes by providing a biofilm matrix, which can act as a sponge to trap and concentrate nutrients in flowing liquids (Costerton 1985). When nutrients are scarce, the bacterial cells within the biofilm are at an advantage relative to those in the bulk liquid flowing past (Zobell 1943).

Exopolysaccharides (EPS) make up a substantial component of microbial exopolymers. In the oceans, EPS exuded by phytoplankton and bacteria coalesce to form transparent exopolymer particles (TEP), which range in size from microns to hundreds of microns (Passow and Alldredge 1994). The aggregation of TEP, phytoplankton, bacteria, fecal pellets, zooplankton and other organic debris form larger particles (> 0.5 mm in diameter), which are known as marine snow (Fowler and Knauer 1986, Silver and Gowing 1991). Marine snow has been shown to include highly concentrated and diverse (Rath et al. 1998) microbial communities engaged in photosynthesis, microbial decomposition (Biddanda 1988) and remineralization of carbon at elevated levels relative to the surrounding seawater (Alldredge and Silver 1988, Simon et al. 1990, Smith et al. 1992). Marine snow particles therefore make a significant contribution to the carbon cycle in the euphotic zone and to the biological pump, which transports fixed carbon to deep waters (Alldredge 2000).

Trace metal enrichment experiments, conducted in ship-board ultra-clean facilities showed that iron may be the most important trace metal controlling phytoplankton development in the Southern Ocean (Scharek et al. 1997). This theory has recently been confirmed by field experiments, including with participation of Australian

scientists (T. Trull, personal communication), where iron was added to a patch of seawater (Boyd et al. 2000). Geider (1999) argued that organic ligands produced by microbes keep iron in solution and that microbes are important in the conversion of iron from the particulate to the dissolved phase. As iron is essential for nitrogen fixation, photosynthesis and respiration, the importance of these microbially produced organic ligands to the biogeochemical iron cycle, and the broader issue of climate change, is worthy of serious consideration and study.

Sea ice is an extreme environment but supports rich and diverse microbial communities that make a major contribution to the global carbon cycle (Bowman et al. 1997a). Microbial EPS appears to be abundant although very little is known about its role in this habitat (Krembs et al. 2002).

1.2. Research Objectives

Obtaining answers to the following questions served as objectives to gain insight into the role of bacterial EPS in microbial processes in the Southern Ocean:

- Can EPS-producing bacteria be isolated from particulate organic carbon (marine snow) present in the water column or from the sea ice?
- Which bacteria are responsible for exopolymer production?
- What is the structure of the polymer?
- How is the polymer structure related to its ecological role?
- How do environmental factors such as temperature affect the production of EPS by marine bacteria?
- Do EPS produced by Antarctic marine bacteria have to ability to bind metal cations such as iron?

1.3. Overview of Subsequent Chapters

Chapter 2 was prepared as an invited review (Mancuso Nichols et al. 2005d). The current literature is discussed with respect to marine bacterial exopolysaccharides.

There is an emphasis on EPS production by microbes living in extreme environments, such as deep-sea hydrothermal vents. Studies of EPS produced by microbes from these high-temperature environments have led to new research into the commercial potential of microbial EPS. The Southern Ocean and sea ice provide enormous microbial biodiversity but very little is known about EPS produced from microbiota in these habitats. The existing level of knowledge on this subject is reviewed.

Chapter 3 examines ten microbial isolates from Southern Ocean particulates and sea ice (Mancuso Nichols et al. 2005c). The isolates have been characterized using molecular, chemotaxonomic and classical microbiological techniques. The growth in liquid culture of these ten isolates was followed by the purification of the EPS produced by each strain. Partial chemical characterization was undertaken and the ecological role of these EPS in the Antarctic marine environment is presented.

Chapter 4 studies EPS production by two closely related isolates from the genus *Pseudoalteromonas*, one obtained from Southern Ocean particulate material, the other isolate from sea ice (Mancuso Nichols et al. 2004). The structure of the two EPS produced by these strains is examined in detail. Results from this investigation provide some insight into the potential diversity of microbial EPS from the Antarctic marine environment.

Chapter 5 investigates the effect of growth temperature on EPS production by a psychrotolerant bacterial isolate of sea ice origin (Mancuso Nichols et al. 2005a). Other researchers have shown that in sea ice, very high salinities and low temperatures may impose pressures on microbial growth and survival. The predicted optimum growth temperature for many sea ice strains is often well above *in situ* temperatures. Growth and EPS production by this sea ice strain was compared over a broad temperature range and the implications of findings with respect to microbial survival and growth in the sea ice are discussed.

Chapter 6 describes a new genus and species of EPS-producing bacteria isolated from particulates sampled from the Southern Ocean (Mancuso Nichols et al.

2005b). Phylogenetic, genotypic, chemotaxonomic and phylogenetic evidence is provided for the establishment of a new bacterial taxon.

Chapter 7 investigates the ability of high molecular weight, highly viscous EPS produced by a sea ice isolate to bind the heavy metals, cadmium and copper. This is a first step towards understanding the ecological role of polymers like this one in the sequestration of trace metals such as iron. This study also provides insight into a possible biotechnological application for EPS produced by Antarctic marine bacteria.

Chapter 8 presents conclusions from this research.

Chapter 2. Bacterial exopolysaccharides from extreme marine environments with special consideration of the Southern Ocean, sea ice and deep-sea hydrothermal vents – a review

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2.1. ABSTRACT

Exopolysaccharides (EPS) are high molecular weight carbohydrate polymers and make up a substantial component of the extracellular polymers that surrounds most microbial cells in the marine environment. EPS comprise a large fraction of the reduced carbon reservoir in the ocean and enhance the survival of marine bacteria by influencing the physico-chemical environment around the cell. Microbial EPS are abundant in the Antarctic marine environment, for example in sea ice and ocean particles, where they may assist microbial communities to endure extremes of temperature, salinity and nutrient availability. The microbial biodiversity of Antarctic ecosystems is relatively unexplored. Deep-sea hydrothermal vent environments are characterized by high pressure, temperature and heavy metals. The commercial value of microbial EPS from these habitats has

been established recently. Extreme environments offer novel microbial biodiversity that produce varied and promising EPS. The biotechnological potential of these biopolymers from hydrothermal vent environments as well as from Antarctic marine ecosystems remains largely untapped.

2.2. INTRODUCTION

The turnover of organic matter by marine microorganisms is an important component of the global carbon cycle (Azam 1998). Organic material in the oceans exists in a heterogeneous continuum from dissolved to particulate matter and this patchiness can support high bacterial diversity (Azam et al. 1994). Dissolved organic matter in the oceans represents one of the largest reservoirs of reduced carbon on earth (McCarthy et al. 1996). Polysaccharides make up a substantial part of oceanic organic matter, especially in surface waters (Benner et al. 1992, Leppard 1995, McCarthy et al. 1996). Studies of bacteria growing in marine sediments, aggregates and detrital particles, show that nearly all the cells are surrounded by extracellular polymeric material (Decho 1990, Costerton 1999) and many of these cells are enclosed within adherent biofilms (White 1986).

Various macromolecules, such as polysaccharides, proteins, nucleic acids and lipids, form the architectural matrix in the intracellular space of microbial biofilms and unattached aggregates in the marine environment (Wingender et al. 1999). The polysaccharide component is the most abundant of these macromolecules since it generally represents 40-95% of the extracellular polymeric substances (Flemming and Wingender 2001a). Abundant microbial polysaccharides present in dissolved organic carbon, particulate material or in biofilms are of major significance in the marine environment. This review provides a brief summary of exopolysaccharide (EPS) biosynthesis, structure-function relationships as well as the role of marine bacterial EPS. We then consolidate what is known so far about the EPS produced by bacteria from two distinct environments, each one experiencing extremes of temperature. There is only a small amount of information in the literature, to the authors' knowledge on the structural elucidation of EPS from Antarctic marine bacteria. As the function is directly

related to its chemical structure, structural elucidation will need to be given priority in determining the ecological role of these polysaccharides and establishing whether they are novel or unique. Finally, the commercial potential of EPS produced by bacteria from these two environments is discussed.

2.3. Regulation of EPS production

Exopolysaccharide (EPS) is a term first used by Sutherland (1972) to described high molecular weight carbohydrate polymers produced by many marine bacteria. Since that time, EPS also has been used to indicate a more broadly define extracellular polymeric substances (Wingender et al. 1999). Within the context of this review, EPS will be used as it was originally defined, that is to mean exopolysaccharide.

EPS can be found in the capsular material that closely surrounds the bacterial cell or released into the surrounding environment as dispersed slime with no obvious association to any one particular cell (Sutherland 1982, Decho 1990). In the natural environment, EPS production seems to be essential for survival since most bacteria occur in microbial aggregates whose structural and functional integrity is based on the presence of a matrix of extracellular polymeric substances (Wingender et al. 1999). Analysis of the polysaccharide component of this matrix from specific bacterial members of natural assemblages is difficult due to the low abundance of any one polymer and the complexity of tracing it back to its source (Christensen 1999). The growth of a single strain under controlled conditions is an approach that is used frequently to examine microbial EPS production in order to form theories about the behaviour of these molecules in the natural environment.

2.3.1. Nutrient limitation

Marine microbes grown in laboratory cultures produce EPS in response to limitation of nutrients such as nitrogen, phosphorus, sulfur and potassium (Sutherland 1982). Exopolymer production may be enhanced in response to physical factors such osmotic stress and temperature (Krembs et al. 2002, Junge et al. 2004). The composition of polysaccharide is generally independent of the

nature of the limiting nutrient. When *Pseudomonas* NCIB 11264 was grown in continuous cultures, its polysaccharide varied little in composition irrespective of pH, temperature, nitrogen, carbon or phosphate content of the growth media (Williams and Wimpenny 1978). The yield of this polymer was higher at suboptimal temperatures, high carbon to nitrogen ratios and during stationary phase. A deep sea hydrothermal vent strain of *Alteromonas* produced EPS at the beginning of stationary phase and during nitrogen limitation, suggesting EPS synthesis for this strain was also induced by restricted growth conditions (Samain et al. 1997). In fact, most deep-sea bacterial isolates examined to date, produce EPS under these conditions (Guezennec, personal observation)

Most bacteria use carbohydrates as a carbon and energy source and amino acids or an ammonium salt as a nitrogen source (Sutherland 1982). The composition of EPS and the chemical and physical properties of these biopolymers can vary greatly (Decho 1990), but it is generally independent of the carbon substrate (Sutherland 1982). Uptake of substrate is one of the first limitations on EPS production and the presence of a carbohydrate substrate such as glucose results in optimal EPS yields (Sutherland 1979). Marine strain *Hahella chejuensis* produced the highest EPS yield in batch culture when grown on sucrose (Ko et al. 2000). Marine bacteria may also produce EPS during growth in sea water alone (Decho 1990) and during carbon limitation since many species can make use of non-sugar sources for EPS biosynthesis (Sutherland 1979).

2.3.2. Growth phase

Batch cultures of a deep-sea hydrothermal vent strain of *Alteromonas* showed stimulated EPS production during nitrogen limited stationary phase (Samain et al. 1997). Most bacteria release the largest quantity of EPS during stationary growth phase in laboratory culture (Decho 1990, Manca et al. 1996). However, a study by Bozal (1994) examined EPS production by a *Pseudoalteromonas antarctica* NF_3 isolated from glacial marine sludge sampled near the South Shetland Islands and found maximum production occurred only during exponential phase. The composition of EPS may also vary according to the growth phase of the bacteria

(Christensen et al. 1985). Although culture conditions generally do not affect the types of monosaccharides in an EPS, they impact on the functional properties of the polysaccharide such as molecular weight, conformation and monosaccharide ratios (Arias et al. 2003). In natural systems where nutrients levels in close proximity to the bacterial cell may vary considerably, shifts in the physiological state of the cell probably result in variable EPS compositions (Geesey 1982).

2.4. BIOSYNTHESIS

One of the first steps in the biosynthesis of EPS takes place when the substrate enters the cell unaltered or after phosphorylation (Sutherland 1977). EPS is synthesized near the cytoplasmic membrane using activated precursors and carrier molecules. Uridine diphosphate-glucose pyrophosphorylase is a key enzyme producing a precursor for both cell wall polymers and exopolysaccharide biosynthesis in many organisms (Sutherland 1977). Several enzymes involved in nucleotide synthesis are membrane bound. Therefore, it is not clear whether their products occur freely within the cytoplasm or whether they are produced in close proximity to the enzymes that require them for polymer synthesis (Sutherland 1977).

The construction of the repeating units is dependent on the transfer of the appropriate monosaccharides from sugar nucleotides to a carrier lipid–isoprenoid alcohol phosphate. The requirement for this carrier lipid in exopolysaccharides biosynthesis is also common to other polymers containing glycan repeating-units located external to the cell membrane including peptidoglycan, teichoic acids and lipopolysaccharides (Sutherland 1977, Sutherland 1982). After polymerisation, the polysaccharide chain may be hydrolysed from the isoprenoid carrier lipid by a highly specific enzyme to produce slime (Sutherland 1977). At the same time, the polysaccharide is transported through the inner and outer membranes (Sutherland 1982). In capsule-producing strains, a ligase reaction may remove the polymer chain from the carrier lipid and attach it covalently to an outer membrane protein (Sutherland 1982) or to phospholipid or lipid-A molecules on the cell surface (Roberts 1996). Capsular exopolymer may only be loosely attached (Costerton et

al. 1992) to the peptidoglycan layer of the cell wall via S-layers, non-covalently associated proteins or glycoproteins cell and can be shed as amorphous slime (Sidhu and Olsen 1997).

2.5. STRUCTURE-FUNCTION RELATIONSHIPS

Most EPS produced by marine bacteria are heteropolysaccharides consisting of three of four different monosaccharides arranged in groups of ten or less to form repeating units (Decho 1990). The monosaccharides may be pentoses, hexoses, amino sugars or uronic acids. Most polymers are linear overall and of varying lengths with an average molecular weight of 100 to 300 kDa (Sutherland 1977). Branches of one or more monosaccharides are often attached at regular intervals (Decho 1990). Organic or inorganic substituents may also be present. Components most commonly found in marine EPS are listed in Table 2.1 (adapted from Kenne and Lindberg 1983).

Table 2.1 Sugar and non-sugar components of bacterial exopolysaccharide*

Type	Component	Example	Mode of linkage
Sugar	Pentoses	D-Arabinose	
		D-Ribose	
		D-Xylose	
	Hexoses	D-Glucose	
		D-Mannose	
		D-Galactose	
		D-Allose	
		L-Rhamnose (6-Deoxy-L-mannose)	
		L-Fucose (6-Deoxy-L-galactose)	
	Amino sugars	D-Glucosamine (2-Amino-2-deoxy-D-glucose)	
		D-Galactosamine (2-Amino-2-deoxy-D-galactose)	
	Uronic acids	D-Glucuronic acid	
		D-Galacturonic acid	
Non-sugar	Acetic acid		*O*-Acyl, *N*-Acyl
	Succinic acid		O-Acyl
	Pyruvic acid		Acetal
	Phosphoric acid		Ester, diester
	Sulfuric acid		Ester

* adapted from Kenne L, Lindberg B (1983) Bacterial Polysaccharides. In: Aspinall GO (ed) The Polysaccharides. Academic Press, New York, pp 287-363

2.5.1. Influence of functional groups

The frequency and type of functional groups present in the EPS impact on the tertiary structure and over-all physico-chemical characteristic of the polymer in the surrounding aqueous environment (Decho 1990). Exopolymers are highly hydrated molecules (up to 99% water, Decho 1990, Sutherland 1977). EPS possess hydroxyl and carboxyl groups, which can have a hydrophilic character in aqueous solutions. EPS produced by marine bacteria may contain up to 20-50% of their EPS as uronic acids (pK_a 3.2-3.4, Kennedy and Sutherland 1987). These are carboxylated sugars and they confer a net negative charge and acidic properties to the EPS (Corpe 1970) at the pH of seawater (pH ~ 8). Depending on their interaction with other organic and inorganic material in the marine environment, microbial exopolymer may be present in dissolved form or as biofilms and aggregates in a gel-like slime matrix (Flemming et al. 1997). Three types of weak interactions provide cohesive forces, and these include: dispersion forces, electrostatic interactions and hydrogen bonding. Weak interactions are significant when the frequency of the functional groups involved and the size of the polymers are considered (Flemming et al. 1997).

2.5.2. Phylogenetic similarities and differences

Taxonomic relatedness does not necessarily ensure similarity of EPS structure. Analysis of 32 closely related *Halomonas* strains, isolated from a hypersaline environment, showed that when grown under the same conditions, EPS yield, chemical composition and physical properties varied from strain to strain (Bouchotroch et al. 2000). The EPS produced by several deep-sea hydrothermal vent bacterial isolates have been well characterized (Rougeaux et al. 1996), including two from genus the *Pseudoalteromonas*. Despite the strains belonging to two different species, the EPS produced by these deep-sea isolates were very similar with respect to crude chemical and monosaccharide composition. Sulfate content was noted as the only structural difference between the polymers in these two hydrothermal vent strains and this component may have influenced the intrinsic viscosity, which also varied (Table 2.2).

The results noted above contrast with those presented in other studies (Mancuso Nichols et al. 2004, Mancuso Nichols et al. 2005c) in which EPS produced by closely related Antarctic strains in the genus *Pseudoalteromonas* vary substantially in terms of crude chemical composition. Another study found two closely related hydrothermal vent bacteria from different subspecies of *Alteromonas macleodii*, and isolated from different sites, produced very different EPS under the same growth conditions (Cambon-Bonavita et al. 2002). These EPS show a high metal binding capacity (Loaec et al. 1998) and are thought to aid in attaching bacteria to the hydrothermal chimney as well as lowering the concentrations of toxic heavy metals in the microenvironment (Table 2.2). EPS produced by the Antarctic marine isolates examined by Mancuso Nichols et al. (2004, 2005c), included carboxyl groups present in uronic acids, amides present in amino sugars, sulfates and hydroxyl groups, which are abundant in all monosaccharides. The authors suggested metal binding as one potential ecological role for these polymers (Table 2.2).

Table 2.2. Examples of characterized marine bacterial exopolysaccharides from Antarctic and deep-sea hydrothermal vent sources

Microorganism	Source environment	Description of EPS	Distinguishing characteristics	Suggested ecological role	Biotechnological application	Reference
Pseudoalteromonas sp. (strain CAM025, bacteria)	Filtered sea ice particulates, **Antarctica**	Sulfated heteropolysaccharide, high in uronic acids with acetyl groups	High* molecular weight (5700 kDa)	Cryprotection in sea ice brine channels	-^	Mancuso Nichols et al 2004
Pseudoalteromonas sp. (strain CAM036, bacteria)	particulates from **Southern Ocean**	Sulfated heteropolysaccharide, high in uronic acids with acetyl and succinyl groups	High* molecular weight (1700 kDa)	Trace metal binding in iron depleted Southern Ocean	-	Mancuso Nichols et al 2004
HYD-1545 (bacteria)	Tissue of marine polychaete from **deep-sea hydrothermal vent (HTV)** habitat	Sulfated heteropolysaccharide, high in uronic acids, with pyruvate	High uptake of heavy metals	-	-	Vincent et al 1994
Alteromonas macleodii subsp. *fijiensis* (bacteria)	Seawater, **deep-sea HTV**, North Fijian Basin	Sulfated heteropolysaccharide, high in uronic acids, with pyruvate	High uptake of lead, cadmium and zinc	-	Thickening agent in food-processing industry, biodetoxification and wastewater treatment, bone healing, treatment of cardio-vascular deseases	Rougeaux et al 1996, Loaec et al. 1997, Colliec-Jouit et al 2001
Pseudoalteromonas sp. (strain GY 768, similar to *P. carrageenovora,* bacteria)	Invertebrate tissues, **deep-sea HTV**, Guaymus Basin	Sulfated (13%) heteropolysaccharide, high in uronic acids, with pyruvate and acetate	Polyelectrolyte character	-	Biodetoxification and wastewater treatment, bone healing	Rougeaux et al 1996, Zanchetta and Guezenec 2001
Pseudoalteromonas sp. (strain GY 786, similar to *P. undina, bacteria*)	Invertebrate tissues, **deep-sea HTV**, Guaymus Basin	Sulfated (6.5%) heteropolysaccharide, high in uronic acids, with pyruvate and acetate	Polyelectrolyte character	-	Biodetoxification and wastewater treatment	Rougeaux et al 1996
Vibrio sp (bacteria)	Invertebrate tissues, **deep-sea HTV** 9°N East Pacific Rise	Heteropolysaccharide high in uronic acids and amino sugars, traces of neutral sugars (EPS 800)	Similar to heparin	-	Anticoagulant activity, anti HIV activity, pharmaceutical activity	Rougeaux et al. 1996
Alteromonas infernus (strain GY 685, bacteria)	Seawater from *Riftia pachyptila*, **deep-sea HTV,** Guaymas Basin	Two EPS, EPS-1 associated with cells, rich in uronic acid and protein; EPS-2. heteropolysaccharide with uronic acids	-	-	Biodetoxification and wastewater treatment	Raguénès et al. 1997

* average molecular weight of most marine bacterial EPS: 100 - 300 kDa (Decho et al 1990)
^ none mentioned

2.6. Roles in the Marine Environment

Exopolymer production may require an energy expenditure of up to 70% and this amounts to a significant carbon and energy investment for the bacterial cell (Harder and Dijkhuizen 1983, Wolfaardt et al. 1999). However, benefits derived from exopolymer production enhance the growth and survival of microbes and the complex communities in which they are found (Wolfaardt et al. 1999). Extracellular polymers augment the ability of microbes to compete and survive in changing environmental conditions by altering the physical and biogeochemical micro-environment around the cell (Costerton 1974). In the marine environment, bacterial exopolymers and EPS are essential in the production of aggregates (Biddanda 1985, Harris and Mitchell 1973, Alldredge and Silver 1988), adhesion to surfaces and other organisms (Marshall 1985, Fletcher and Floodgate 1973, Paerl 1975, Paerl 1976, Vincent et al. 1994, Holmstrom and Kjelleberg 1999), biofilm formation (Sutherland 2001, Sutherland 1999), sequestering of nutrients (Decho and Herndl 1995), as well as providing protection (Decho and Lopez 1993, Bitton and Friehofer 1978) and ecosystem stability (Uhlinger and White 1983, Dade et al. 1990). The role of microbial exopolymers in the ocean has been reviewed extensively (Decho 1990, Wolfaardt et al. 1999) and is summarized briefly below and in Table 2.3. Where information relates to EPS, specific mention is made.

2.6.1. Adhesion to and colonization of surfaces.

Surfaces exposed to seawater quickly adsorb and concentrate dissolved organic compounds. Attachment to these surfaces by bacteria provides the opportunity for growth in dilute solutions that would otherwise be unavailable (Zobell 1943, Paerl 1975). Charged substrates including amino acid, sugars, fatty acids and glycoproteins are often the first concentrated on surfaces (Marshall 1985). Many bacterial cells possess a capsule prior to attachment. Capsular and slime

Table 2.3. Some of the roles of microbial exopolymeric material in the marine environment

Role of exopolymer	Examples	References
Assists in attachment to surfaces	Exopolymers of marine *Vibrio* MH3 were involved in reversible attachment	Hermannson and Marshall 1985
	Cross-linking of adjacent polysaccharide chains aided in permanent adhesion	Marshall 1980
Facilitates biochemical interactions between cells	Exopolymer matrix localized secreted exoenzymes	Decho and Herndl 1995
	Exopolymer mediate bacterial attachment to the polar end of bluegreen N2-fixing alga. EPS aided attachment to symbiotic host such as vent tube worm to absorb metals and detoxify microenvironment.	Paerl 1974, Vincent et al. 1994
	Exopolymer buffered against sudden osmotic changes	Dudman 1977
Provides protective barrier around the cell.	Bacteria in aggregates were less preferred by grazers that freely suspended bacteria	Caron 1987
	EPS-producing deep-sea hydrothermal vent bacteria showed resistance to heavy metal. Metal binding involves cell wall components as well as polysaccharides	Jeanthon and Pieur 1990, Spath et al. 1998
	Exopolymer in sea-ice brine channels provided cryoprotection by interacting with water at low temperature to depress freezing point	Krembs et al. 2002
	Nutrient uptake by bacteria in aggregates was higher than for free-living cells in low nutrient systems	Logan and Hunt 1987
Absorbs dissolved organic material	Porous and hydrated matrix acts like a sponge and sequestered and concentrated dissolved organics	Decho 1990, Decho and Lopez 1993

heteropolysaccharides that contain uronic acids (pKa ~ 3) confer a net negative charge to the cell above pH 3 (Sutherland 1980). Since surfaces and cells both tend to be anionic, the presence of positive ions such as Ca^{2+} is important.

Exopolymers including capsular polysaccharides and proteins are important in bacterial adhesion to surfaces (Wolfaardt et al. 1999). The initial attachment can be reversible and is also related to the electrostatic interactions and cell wall hydrophobicity (van Loosdrecht et al. 1990, van Loosdrecht et al. 1987). Irreversible binding may occur since some bacteria, in close proximity to a surface, secrete large amounts of EPS-slime (Costerton 1984). Additional cross-linking of adjacent EPS chains enable permanent attachment to occur (Marshall 1980). This process is influenced by electrolyte concentration (Fletcher 1988). Bacteria may reversibly attach by secreting an exopolymer allowing them to stick to a surface and use surface-associated nutrients (Hermannson and Marshall 1985). This is followed by the secretion of a second polymer, which releases the attached bacteria.

2.6.2. Facilitates biochemical interactions

Biochemical interactions between the bacteria and surrounding cells and tissues may be made possible by exopolymer material (Logan and Hunt 1987, Decho 1990). Exopolymer slime and capsular material provide a biofilm matrix around the cell. This is a hydrated layer, which can provide a buffering against sudden changes in the adjacent osmotic environment (Dudman 1977). Such a stable environment may aid in the localisation of secreted exoenzymes, which are essential in the cycling of both organic and inorganic material in the marine environment (Decho 1990). The hydrated exopolymer matrix retains the exoenzymes activity in close proximity to the cell. This facilitates cellular uptake of small molecules for metabolic conversion to energy and biomass (Decho and Herndl 1995).

Symbiotic relationships may also occur between bacteria and other organisms. Bacteria adhere to the site of nitrogen fixation on cyanobacterial heterocysts (Paerl

1976) via the heterocyst-produced EPS (Lupton and Marshall 1984). These microzones around cells facilitate the transfer of nutrients from one organism to another (Paerl 1976). The heavy metal binding properties of an EPS produced by a hydrothermal vent strain was thought to be advantageous to the tubeworm host. (Vincent et al. 1994). Members of the genera *Pseudomonas* and *Alteromonas* produce polysaccharide-containing exopolymers that potentially benefit the survival of other marine organisms by facilitating attachment to surfaces (Szewzyk et al. 1991, Holmstrom and Kjelleberg 1999).

2.6.3. Provides a protective barrier around the cell.

Exopolymer may act as a physical barrier to grazers. In a study by Caron (1987), microflagellates grazed more readily on freely suspended bacteria than on those on surfaces or enclosed in aggregates. The EPS may have provided protection to cells within the aggregates, since the microflagellates were only able to graze the bacteria on the surface of the aggregates. Slime exopolymer from one bacterial strain may be preferred by consumers to the capsular exopolymer of the same strain (Decho and Lopez 1993).

Changes in pH and salinity over a wide range had little effect on the viscosity and stability of EPS produced by marine bacteria in a study by Boyle and Reade (1983). Such results suggest that these EPS may provide some buffering against shifting environmental conditions in the natural environment. Bacteria isolated from deep-sea hydrothermal vent communities displayed resistance to heavy metals (Jeanthon and Prieur 1990) and the purified EPS produced by these strains in laboratory cultures showed very good metal binding properties (Loaec et al. 1997, Loaec et al. 1998). Capsular polysaccharide may also provide the bacterial cells with a protective barrier against toxic substances in the water column (Bitton and Friehofer 1978).

In biofilm studies involving removal of organic and heavy metal pollutants, exopolymeric substances removed the majority of organic pollutants while heavy metals were taken up by the cellular fraction. These results indicated an important role for cell wall components such as proteins in metal binding in complex biofilm

systems (Spaeth et al. 1998). These findings were confirmed in a more recent study that showed heavy metals were bound by cellular sorption as well as extracellularly by polymeric substances such as polysaccharides in bacterial biofilms and microbial flocs (Wuertz et al. 2000).

Cells imbedded in the gel matrix of a biofilm are well protected from biocidal treatments (Brown and Gilbert 1993, McBain and Gilbert 2001). Current strategies to eliminate unwanted biofilms involve the design of antimicrobial agents that can penetrate the gel matrix and target slow or dormant cells. Some success has been achieved by incorporating transition metal catalysts into the substratum. These generated biocidal species and killed the biofilm from the inside overcoming the protection provided by the exopolymer matrix (Wood et al. 1998).

2.6.4. Acts as a sponge for sequestering dissolved organic material.

In natural aquatic environments, the nutrients required to support maximal microbial growth rarely are present in sufficient quantities in the water column. Microbial attachment to fixed surfaces, other cells and aggregates is a likely strategy to increase the rate of substrate uptake (Logan and Hunt 1987). Microbial cells surrounded by a porous matrix of exopolymer sequester and concentrate dissolved organic compounds (Decho and Lopez 1993). The highly hydrated exopolymer matrix act as a sponge to trap and concentrate nutrients in flowing liquids (Decho 1990).

2.7. EPS IN THE ANTARCTIC MARINE ENVIRONMENT

2.7.1. Antarctic sea ice

2.7.1.1. Bacteria and exopolymers are abundant

Bacteria contribute significantly to secondary production in sea ice communities and to the overall carbon cycle in the Antarctic environment. Annual sea-ice is a microhabitat for a complex community of marine bacteria often in close association with microalgae. These assemblages are essential components of

carbon and energy transfers in the Southern Ocean (Sullivan and Palmisano 1984). Abundant bacterial populations have been found in thick annual pack ice, with psychrophilic bacteria being particularly common in samples of brown ice and pore waters (Delille 1992). Studies of both the Arctic (Krembs and Engel 2001) and Antarctic (Sullivan and Palmisano 1984) sea-ice communities suggest that exopolymer production by both microalgae and bacteria contribute to organic carbon in the sea-ice and ice-water interface. In thick pack ice, bacterial secondary production even exceeds primary production as the light supply to the bottoms layers of ice is reduced (Grossmann and Dieckmann 1994).

Sea ice bacteria maintained in laboratory culture secreted large amounts of mucous (Helmke and Weyland 1995). In a more recent *in situ* study of bacterial-algal interactions in melting sea ice in the Weddel Sea, it was suggested that bacterial mucous contributed to particulate organic carbon sustaining microbial growth in the sea ice crack pools (Gleitz et al. 1996). Bacterially-produced EPS may provide a means by which bacteria can adhere to the microalgal cells (Sullivan and Palmisano 1984). During ice formation, microalgal cells are scavenged by sea ice crystals floating up to the sea surface (Gleitz and Thomas 1993) and bacteria attached to algal cells may be incorporated into new ice in conjunction with some algal species (Grossmann and Dieckmann 1994).

2.7.1.2. High salinity, low temperature environments

Bacteria are found in abundance in the bottom layers of the ice or in brine channels, and are often attached to detrital particles or to living microalgal cells (Sullivan and Palmisano 1984, Archer et al. 1996, Delille 1996). Delille and Rosier (1996) also suggested that the high numbers of particle associated bacteria found in sea ice may explain observations of underlying seawater being enriched in bacterial biomass relative to the open ocean (Grossmann and Dieckmann 1994). More recently, studies of Arctic sea ice in winter showed that even at temperatures as low as –20°C and salinity of 209 ppt, active bacteria were found in the brine channels and were particle associated (Junge et al. 2004). The same authors showed that high concentrations of exopolymeric substances were found in brine

channels and could have been produced by the abundant bacteria or diatom populations present.

A study of Arctic sea ice demonstrated that photosynthesis rates by phytoplankton from under the ice were stimulated to similar levels by sea ice extracts as they were by the chelator, ethylenediamine tetra-acetic acid, and trace metals (Apollonio et al. 2002). From those results, the authors suggested that a natural 'conditioning agent' is produced within the bottom-ice algal layer that enhances phytoplankton growth. Sea ice bacterial communities and high amounts of exopolymer are concentrated in these layers (Krembs and Engel 2001, Krembs et al. 2002). It is yet not clear that in addition to the availability of sufficient light, whether the abundance of trace metals is a limiting nutrient for primary production in sea ice microbial communities.

2.7.1.3. EPS as a cryoprotectant

Arctic studies (Krembs and Engel 2001, Krembs et al. 2002) have shown that large quantities of microbially produced exopolymeric substances occur in sea ice and at the ice-water interface. This material was positively correlated to bacterial abundances, although diatoms were thought to dominate the exopolymer production in this system. These authors suggested high concentrations of exopolymer with its high polyhydroxyl content would decrease the freezing point of water in the low temperature, high salinity brine channels, especially near the cell, where concentration of exopolymer are highest (Krembs et al. 2002). Exopolymer in the brine channels might have provided buffering against harsh winter conditions and high salinity as well as cryoprotecting the microbes living there against ice crystal formation by depressing the ice nucleation temperature of water (Krembs et al. 2002).

In a recent study (Mancuso Nichols et al. 2005c), EPS produced by sea ice isolates were shown, by molecular weight analysis to be between 5 and 50 times larger than the average observed for other marine EPS (100 - 300 kDa, Decho 1990). The structure and properties of EPS are influenced by the length of the polymer chain, that is the molecular weight (Christensen 1999). As the length of the

polymer increases, there is a greater opportunity for complex entanglement of the chains and intramolecular associations, and these contribute to the tertiary structure and physical behavior of the polymer (Sutherland 1994). A fungal strain, *Phoma herbarum,* isolated from Antarctic soil produced a homosaccharide of glucose with a molecular weight of 7400 kDa (Selbmann et al. 2002). The authors of this study suggested that the fungal EPS could have provided a cryoprotective role in the harsh Antarctic environment where the availability of liquid water and temperatures were extremely low. Similarly, the freezing processes in sea ice result in brine channels where temperature is very low and salinity is high due to brine. EPS may be providing a cryoprotectant role in these environments of high salinities and low temperature (Krembs et al. 2002)

In a study by Mancuso Nichols et al. (2004), a strain of Antarctic *Pseudoalteromonas* isolated from sea ice, produced 30 times as much EPS at -2 and 10°C compared to 20°C in liquid culture. Previous studies have shown that many *Pseudoalteromonas* strains are psychrotrophic bacteria with a temperature growth range from 4°C to 30°C (Bozal et al. 1997, Bowman 1998), and show optimal growth at 22°C to 25°C (Bowman 1998). Members of this genus are among the dominant bacteria generally found in this environment as determined by cultivation dependent and independent techniques (Bowman et al. 1997c, Staley and Gosink 1999, Brown and Bowman 2001, Brinkmeyer et al. 2003). In the Mancuso Nichols et al. (2004) study, the consumption of glucose per mg of EPS produced was highest at -2°C, well below the expected optimal growth temperature for this genus (20°C). This finding supports the proposed hypothesis that EPS production by psychrotolerant bacteria may play an important role in the sea ice microbial community. Whether this increased EPS production at low temperature is a specific cold adaptation mechanism for this strain requires further investigation. Bacterial EPS production in brine channels and perhaps other cold, high salinity ecosystems may provide a barrier against the environmental extremes experienced by the bacterial cell by modifying water properties near the cell.

Arctic sea ice studies (Krembs and Engel 2001, Krembs et al. 2002) also demonstrated that the neutrally buoyant polymeric material was carried large

distances by prevailing under-ice currents and ice drifts. Studies in more temperate waters showed marine bacterial exopolymer production was important in the aggregate formation process (Biddanda 1986, Decho 1990). When released into the water column, a combination of biological, chemical and physical forces caused this colloidal material to form aggregates (Alldredge and Jackson 1995, Passow 2000, Kiorboe 2001), which became centers of high microbiological heterotrophic activity (Kiorboe 2001).

2.7.2. EPS in the Southern Ocean

2.7.2.1. Marine Snow

In natural aquatic systems, when exopolymer is associated with particulate material, it exists in particulate form, or it is present in its colloidal form, which is operationally defined as part of the dissolved organic matter (DOM) because it can pass through a filter with a given pore size (less than 0.1 μm to 0.46 μm, Chin et al. 1998). In the oceans, exopolymer exuded by phytoplankton and bacteria coalesce to form transparent exopolymer particles (TEP) that range in size from microns to hundreds of microns (Sullivan and Palmisano 1984, Passow and Alldredge 1994). The aggregation of TEP, phytoplankton, bacteria, faecal pellets, zooplankton and other organic debris form larger particles (>0.5 mm in diameter), which are known as marine snow (Fowler and Knauer 1986, Logan and Hunt 1987, Mueller-Niklas et al. 1994).

2.7.2.2. Marine snow in the Southern Ocean

Marine snow has been shown to include highly concentrated and diverse microbial communities (Rath et al. 1998) engaged in photosynthesis, microbial decomposition (Biddanda and Pomeroy 1988, Biddanda 1988) and remineralization of carbon at elevated levels relative to the surrounding sea water (Alldredge and Silver 1988, Simon et al. 1990, Smith et al. 1992). Marine snow particles therefore make a significant contribution to the carbon cycle in the euphotic zone and to the 'biological pump', which transports fixed carbon to deep waters (Alldredge 2000, Kiorboe 2001). The flux of aggregates in the Ross Sea, near the Antarctic peninsula, was found to dominate the vertical export of organic

matter from the euphotic zone (Asper and Smith 2003). In another study, the abundance of marine snow particles in samples taken near Australian Antarctic bases Davis and Mawson was approximately 100 times lower that observed in the Ross Sea (Marchant et al. 2000). Spatial and temporal variation in particle production and sedimentation has been observed previously in the Antarctic marine environment (Karl et al. 1991). This variation was also consistent with findings from temperate and tropical waters (Alldredge and Silver 1988).

Bacteria in marine aggregates are at an advantage compared to free living cells (Logan and Hunt 1987). Their proximity to other cells and surfaces provides opportunities for interaction and nutrient uptake. Bacterial polysaccharides form the fibrillar frame work, act as glue in the ultrastructure and provide the structural network for microbial associations within marine aggregates (Biddanda 1986, Decho and Herndl 1995, Heissenberger et al. 1996, Lewis 2000, Flemming and Wingender 2001a). Microscopic and laboratory studies have shown that bacterially produced EPS have a major role in aggregate formation (Biddanda 1986, Heissenberger and Herndl 1994, Leppard 1995).

2.7.2.3. EPS as organic ligands

The ability of bacterial EPS to accumulate metals has been known for some time (Bitton and Friehofer 1978, Brown and Lester 1979, Loaec et al. 1997) and at the pH of ambient seawater (pH ≈ 8), anionically charged EPS can remove >99% of Zn and Ag (Harvey and Luoma 1985). Exopolymer complexation with trace metals may impact strongly on the availability of these micronutrients to marine organisms and may be important in the downward transport of trace metals and micronutrients in the ocean (Decho 1990). Microbial EPS may also be a major component of the colloidal matter which has been proposed to bind trace metals within an 'onion'-like matrix of metal oxides/hydroxides and organic compounds (Mackey and Zirino 1994).

Most (99%) dissolved iron in the ocean is bound to organic ligands with a high affinity for iron (Rue and Bruland 1995). Wu et al.(2001) examined the soluble and colloidal iron in the oligotrophic North Atlantic and North Pacific and showed

that soluble iron and iron-binding organic ligands were depleted at the surface and enriched at depth. The authors suggested that iron, which was once thought to be dissolved and available to phytoplankton, might be tied up as colloidal material, which eventually aggregates and settles out of the photosynthetic zone. In another study in the subarctic Pacific Ocean, Maldonado and Price (1999) showed that heterotrophic bacteria play a significant role in dissolved iron uptake and that the iron bound to strong organic ligands, the most predominant form of iron in the sea, is available to phytoplankton in these environments.

Trace metal enrichment experiments, conducted in ship-board ultra-clean facilities showed that iron may be the most important trace metal controlling phytoplankton development in the Southern Ocean (Scharek et al. 1997) and this has recently been confirmed by field experiments where iron was added to a patch of seawater (Boyd et al. 2000). The iron-stimulated diatom bloom was succeeded by an increase in diatom-associated silica particulates in sediment traps at depth after three weeks. This was followed by increased particulate organic carbon exported to deeper waters. Bacterial remineralization and mesozooplankton grazing of this particulate material, which may have included exopolymer material, accounted for over half the increased particulates associated with addition of iron to the system. Geider (1999) argued organic ligands produced by microbes keep iron in solution and that microbes are important in the conversion of iron from the particulate to the dissolved phase. As iron is essential for nitrogen fixation, photosynthesis and respiration, the importance in these microbially produced organic ligands to the biogeochemical iron cycle and the broader issue of climate change should not be overlooked.

2.7.2.4. Implications for primary chemical structure

Although actual content may vary based upon extraction and purification method used (Neilsen and Andreas 1999), exopolysaccharides produced by marine bacteria may contain up to 20-50% of the polysaccharide as uronic acid (Kennedy and Sutherland 1987). The presence of uronic acids contributes a negative charge to the overall polymer (Decho 1990). Sulfate was thought to occur only in polysaccharides produced by Archaea and Cyanobacteria until recently, and its

presence in polymers produced by prokaryotes is seen as uncommon (Arias et al. 2003). When sulfate is present as a functional group, it also contributes to the anionic quality of these EPS in seawater (Leppard et al. 1996). The overall negative charge gives the molecule a 'sticky' quality. This anionic charge is important in terms of the affinity of these EPS for binding to cations such as dissolved metals (Brown and Lester 1982).

Korstgens et al (2001) studied biofilms formed by *Pseudomonas aeruginosa* that were dominated by polysaccharides with carboxyl groups. Calcium, copper and iron provided stability to the network by acting as bridging ions. The presence of calcium and magnesium salts resulted in increased viscosity in solutions of polysaccharide from marine bacteria stored at low pH (Boyle and Reade 1983). Divalent cations provide stability to the polysaccharide gel matrix (Decho 1990). Recent work with the purified EPS from *Ps. aeruginosa* showed that there were strong electrostatic interactions between divalent cation manganese and the carboxylate groups occurring along the EPS chain. This study provides insight into the molecular geometry of the stability provided by divalent cations such as calcium, which are present in marine systems (Emmerichs et al. 2004).

Bacteria isolated from Southern Ocean particulate material produced EPS in liquid culture (Mancuso Nichols et al. 2004). Preliminary characterizations show that the structure of the EPS includes sulfate as well as high levels of uronic acids as galacturonic acid, along with acetyl groups. In addition, the EPS was shown by NMR data to include a succinyl group. These features convey an overall polyanionic quality to the EPS in the marine environment, since at the pH of seawater many of the acidic groups present on these polymers are ionized (Decho 1990). This 'stickiness' is important in terms of the affinity of these EPS for binding of cations such as dissolved metals (Brown and Lester 1982). The EPS produced by Antarctic bacterial isolates examined by Mancuso Nichols et al. (2005c) appear to be polyanionic and, therefore, 'sticky' with respect to cations such as trace metals. The availability of iron as a trace metal is of critical importance in the Southern Ocean where it is known to limit primary production (Scharek et al. 1997). Since 99% of dissolved iron in the ocean is bound to

organic ligands (Rue and Bruland 1995), implications for the role of these bacterial polysaccharides in the Antarctic marine environment require further investigation.

Monosaccharide analyses of the ten EPS produced by Antarctic marine bacteria (Mancuso Nichols et al. 2005c) showed that the sugars present were generally similar to sugars typically found in bacterial EPS (Table 2.1, Kenne and Lindberg 1983) and more specifically in marine bacterial EPS (Kennedy and Sutherland 1987). Arabinose was present, to varying degrees, in all EPS produced by Antarctic bacteria examined in this study and xylose was present as a minor component in several strains. Arabinose and xylose are not commonly found in bacterial EPS but are components of the lipopolysaccharide layer of some microbes (Kenne and Lindberg 1983). The significance of these finding requires further investigation.

An exopolysaccharide, known as mauran, is produced by the halophilic bacterium, *Halomonas maura* (Arias et al. 2003). When this strain was grown in media containing salt (2.5%, w/v), it produced a high molecular weight (4500 kDa) EPS that contained glucose, mannose and galactose as well as high amounts of glucuronic acid (8%, w/w) and sulfates (6.5%, w/w). This polysaccharide was able to bind a range of heavy metal cations. The authors also noted the stability of muran under different conditions of stress including high salt concentrations and during freezing/thawing. There are similarities between muran and several of the EPS produced by the Antarctic marine isolates in terms of chemical composition.

Further research is necessary to more accurately define the structure of the Antarctic marine bacterial EPS and to relate these findings to the function of these molecules in the natural environment. As yet, it is unclear how these polysaccharides may be acting mechanistically as organic ligands, protectants against low temperature or high salinity, or whether the size of these EPS is related to their ecological role. An increased understanding of these structural and functional roles is also a prerequisite to potential biotechnological exploitation of Antarctic bacterial EPS.

2.8. EPS FROM THE DEEP-SEA

2.8.1. Hydrothermal vent communities

The oceans constitute more that 70% of the earth's surface. The deep sea (>1000 m) was once thought to be a biological desert until submarine hydrothermal systems were discovered along mid-ocean ridges at depths greater than 2200 m (Snelgrove and Grassle 1995). Geological formations include hot fumaroles, springs and sediments and deep-sea vents (Stetter 1998). In these environments, hydrostatic pressure averages 25 x 10^6 Pascals, and temperatures can range from 380°C within the fumarole to 2°C in the surrounding sea-water (Yayanos 1998). Hot anaerobic waters rich in hydrogen sulfide and heavy metals escape the vents and blend with cold oxygenated seawater. The presence of heavy metals is a characteristic feature of the hydrothermal vent environment (Jeanthon and Prieur 1990). Despite these environmental extremes, a complex food web based on chemosynthesis including dense invertebrate populations supported by a rich microbial community of heterotrophic and autotrophic bacteria were found in the vicinity of the vents (Antoine et al. 1995).

The selective pressures imparted on the inhabitants of this ecosystem result in hydrothermal vent environments being centres of unusual biological communities. Vent environments are now considered to be an enormous source of genetic and metabolic microbial biodiversity (Deming and Baross 1998). Innovations in microbiological culturing techniques recently have been employed to gain insight into microbial biochemical processes and microbial by-products used for growth and survival in these hydrothermal vent environments (Deming 1998).

2.8.2. Microbial EPS from hydrothermal vents

Deep-sea hydrothermal vents offer a new source of a variety of fascinating microorganisms well adapted to these extreme environments. Over the past 17 years, an increasing number of new genera and species of both hyperthermophilic and mesophilic bacteria have been isolated from these vents communities (Guezennec 2002). Bacteria associated with deep-sea hydrothermal vent communities have demonstrated their ability to produce unusual extracellular

polymers in an aerobic carbohydrate-based medium and so far, three main EPS producing genera have been identified *i.e.*, *Pseudoalteromonas*, *Alteromonas* and *Vibrio*. To date, only a small number of EPS have been fully characterized, since they hold some biotechnological promise (see below). Information related to the chemical composition of these polymers reveals potential commercial usefulness at the same time as providing insight into their role in the deep-sea vent ecosystem.

2.9. BIOTECHNOLOGICAL POTENTIAL OF MICROORGANISMS

Biotechnology is recognised as one of the most promising technologies for the 21st century considering its potential to ameliorate major global problems (disease, malnutrition and environmental pollution), achieve industrial sustainability (optimising use of renewable resources, slowing global warming and developing cleaner products and processes) and achieve economic competitiveness (Bull et al. 2000). Since biotechnology is based on the discovery of exploitable biology, the recognition that only a very small fraction of the earth's microbial biodiversity has been identified implies a great potential for innovation. Knowledge of the interaction of microbes in their environment is critical in accessing both the microbe itself and processes it employs to survive, both of which hold biotechnological promise (Bull et al. 2000).

2.9.1. Biotechnological potential of EPS

The species-specific structural heterogeneity and the many roles EPS play in the natural environment are reflected in the numerous existing and potential applications for these bio-polymers (Weiner 1997). Xanthan gum, the most well known microbial polysaccharide, is produced by the plant-pathogen *Xanthomonas campestris* pv. *campestris*. Because of its physical properties it is commonly used as a thickener in both food and non-food industries (Becker et al. 1998). Bacterial cellulose, produced by *Acetobacter xylinum* and other, mainly Gram-negative bacterial species, has a high water binding capacity. This EPS is used to make a type of wound dressing for patients with burns, chronic ulcers or extensive tissue loss (Sutherland 1998). Several *Agrobacterium* and *Rhizobium* species produce

curdlan and this improves the texture of tofu, bean jelly and fish pastes in Japan (Sutherland 1998). The study of EPS produced by bacteria from the marine environment provides additional opportunities for novel uses of these biopolymer

2.9.1.1. EPS from deep-sea hydrothermal vents

In recent years, there has been a growing interest in the isolation and identification of new microbial polysaccharides that may have novel applications such as viscosifiers, gelling agents, emulsifiers, stabilizers and texture enhancers. In the course of the discovery of novel polysaccharides of biotechnological interest, it is now widely accepted that extremophilic microorganisms will provide a valuable resource not only for exploitation in biotechnological processes but also as models for investigating how biomolecules are stabilized when subjected to extreme conditions. Deep-sea hydrothermal vents offer a new source of novel bacteria. Several have been found to produce exopolymers with exploitable properties.

EPS-producing thermophilic and mesophilic strains have been sourced from vent environments and the EPS produced by these strains in laboratory culture have been examined for a range of applications. Several bacterial exopolymers were found to be novel with significant biotechnological potential (Guezennec et al. 1994, Raguénès et al. 1997b). To date, investigations generally have been performed on mesophilic heterotrophic bacteria rather than on thermophilic and hyperthermophilic microorganisms. This is despite the biotechnological appeal of microorganisms adapted to life at high temperature that may produce thermostable enzymes (Guezennec 2002).

The structure of the EPS produced by *Pseudoalteromonas* strain 721 has been investigated. The repeating unit of this polymer shows some irregularities but can be defined as an octasaccharide with two side-chains (Figure 2.1, Rougeaux et al. 1999, Guezennec 2002). This exopolymer exhibits a gelation following thermal treatment. The viscoelastic behaviour of the HYD721/NaCl system under varying temperatures suggests that two effects contribute to the creation of the gel network.

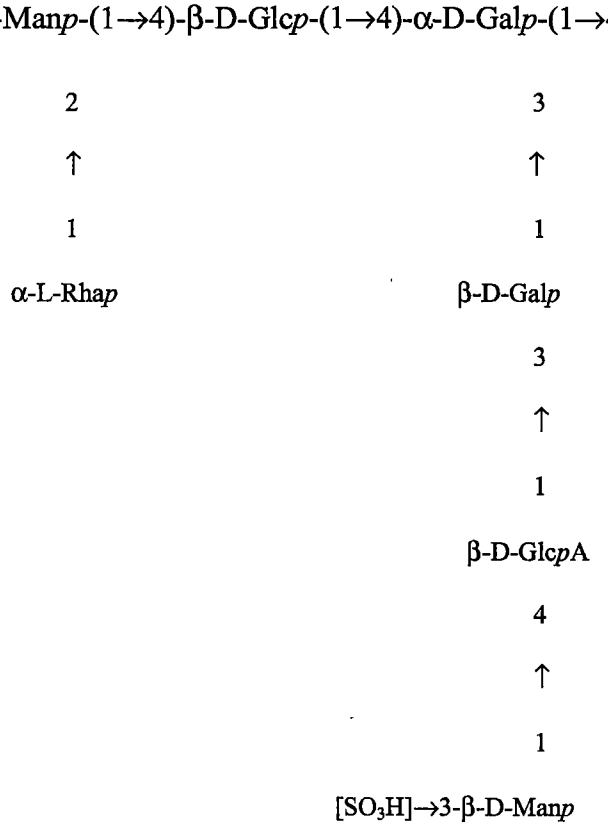

Figure 2.1 Repeating unit of the exopolysaccharide secreted by *Pseudoalteromonas* sp. strain 721.

Intermolecular associations observed with increasing temperature are probably the result of hydrophobic interactions between methyl groups of the rhamnose residues (Guezennec 2002).

Alteromonas strain 1545, isolated near a hydrothermal vent from the epidermis of the polychaete *Alvinella pompejana*, produces an anionic EPS under laboratory conditions (Talmont et al. 1991). A polysaccharide secreted by a bacterium (*Alteromonas* strain 1644) isolated from *Alvinellidae* samples, collected near a hydrothermal vent of the East Pacific Rise showed an original chemical structure and unique rheological behaviour (Figure 2.2, Guezennec 2002). This polymer shows strong selectivity between monovalent and divalent ions and exhibits a great affinity for

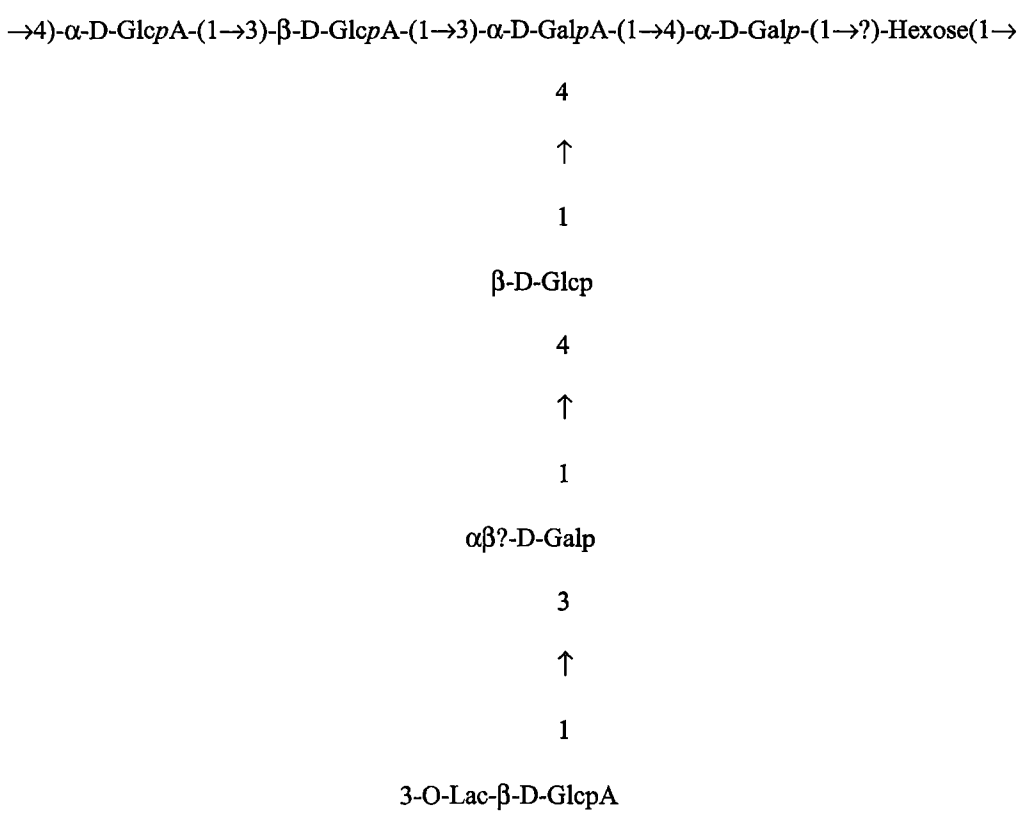

Figure 2.2. Repeating unit of the exopolysaccharide produced by *Alteromonas* sp. strain 1644.

the divalent ions, higher than predicted by electrostatic theories (Bozzi et al. 1996a, Bozzi et al. 1996b) with the exception for Mg^{2+}. *A. macleodii* subsp. *fijiensis* is an aerobic, mesophilic, heterotrophic bacterium isolated from a diluted hydrothermal vent at a depth of 2600m in a rift system of the North Fiji basin (16°59'S, 173°55' W). This strain produces an EPS with a high metal-binding maximum capacity (up to 316 mg Pb(II) / g polymer, Loaec et al. 1997, Loaec et al. 1998). Proposed uses for this polymer include water treatment and removal of heavy metal pollutants (Table 2.2). This EPS also holds promise as a food-thickening agent since it has many chemical similarities to xanthan (Figure 2.3, Rougeaux et al. 1996, Guezennec 2002). This hydrophobic bacterial exopolysaccharide also was shown to encourage adhesion of osteoblastic cells during *in vivo* experiments conducted on rat calvaria. Results suggested that hydrophobic EPS matrix added to bone surfaces might encourage healing (Zanchetta et al. 2003b). Other studies of EPS produced by bacteria from these vent environments either in their native

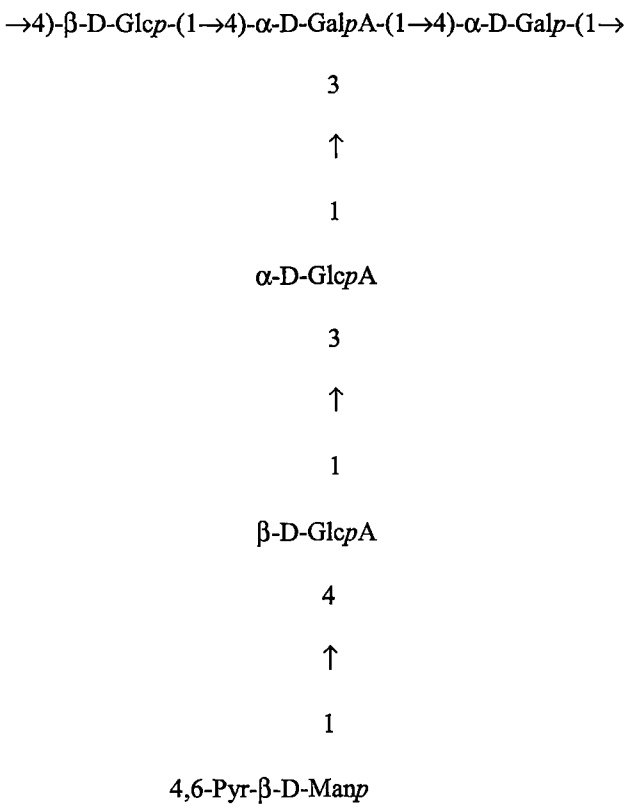

Figure 2.3. Repeating unit of the exopolysaccharide secreted by *Alteromonas. macleodii* subsp. *Fijiensis.*

state or following chemical modifications also suggested clinical applications in the area of cardio-vascular diseases (Colliec-Jouault et al. 2001, Matou et al. submitted) and bone healing.

A facultatively anaerobic, heterotrophic and mesophilic bacterium was also isolated from a Pompeii worm (polychaete *Alvinella pompejana*) tube collected from a deep-sea hydrothermal field of the East Pacific Rise and named *Vibrio diabolicus*. This was the first member of the genus *Vibrio* to be isolated from a deep-sea ecosystem. Novel EPS produced by this strain is characterized by equal amounts of uronic acid and hexosamine (*N*-acetyl glucosamine and *N*-acetyl galactosamine (Figure 2.4, Raguénès et al. 1997a, Guezennec 2002).The role of this novel bacterial polysaccharide in bone regeneration has been recently successfully investigated (Zanchetta et al. 2003a, Zanchetta et al. 2003b).

There is not doubt that extreme environments such as deep-sea hydrothermal vents are a rich source of microorganisms of biotechnological importance. A number of

→3)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*A-(1→4)-α-D-Gal*p*NAc-(1→

Figure 2.4. Repeating unit of the exopolysaccharide secreted by *Vibrio diabolicus.*

interesting and unique polysaccharides have been isolated from these microorganisms and these are expected to find industrial applications in the very near future. Further screenings are underway as well as research into understanding the structure-function relationships of these unusual polymers (Guezennec 2002).

2.9.1.2. Cool prospects: EPS from Antarctica

The Antarctic marine environment is perennially cold, in some cases it is permanently ice covered. Extremes of temperature, salinity, and water activity govern microbial life in enriched microenvironments for example, hypersaline sea ice brine channels, the pelagic water column and particles. Spatial heterogeneity combined with extreme seasonal fluctuations such as those experienced during annual sea ice formation events results in a high diversity of microbial habitats and therefore, microbial communities (Karl 1993). Very few bacterial species isolated from the Antarctic marine environment have been described to date (Nichols et al. 2001). An opportunity clearly exists for the search and discovery of novel microbial products with biotechnological potential.

Ecological studies examining the role of EPS in marine habitats now provide evidence that these substances are abundant in the Antarctic marine environment (Helmke and Weyland 1995, Krembs and Engel 2001, Krembs et al. 2002, Mancuso Nichols et al. 2004, Mancuso Nichols et al. 2005c). However, few studies focusing on the biotechnological potential of EPS produced by bacteria from the Antarctic marine environment are available from the literature to date. *Pseudoalteromonas antarctica* NF_3 produces a exopolymeric compound of glycoprotein character that displays the ability to coat liposomes and provides protection against surfactants (Cocera et al. 2000, Cocera et al. 2001). A study by Mancuso Nichols et al. (2005c) has shown that, even among closely related strains, EPS produced by Antarctic bacteria commonly found in the marine

environment were diverse. The full subunit structure of EPS produced by Antarctic marine bacteria remains to be elucidated. This information will facilitate assessment of possible commercial applications. These initial studies reveal largely untapped reservoir of biotechnological potential is waiting to be accessed. Whether these EPS will become useful as cryoprotectants, chelators of heavy metals or in some other form remains to be established.

2.10. Conclusions

Bacterial exopolymers and their EPS components are abundant and ubiquitous in the marine environment where they serve essential functions that enhance microbial survival. In the Antarctic marine environment, exopolymers may provide cryoprotection in high salinity, low temperature brine channels. In the Southern Ocean iron limits primary productivity and the resulting draw-down of carbon dioxide, an important green house gas, from the atmosphere. Microbial EPS in suspended aggregates of marine snow may influence the availability of dissolved iron for primary production in Antarctic waters. In hydrothermal vent environments of the deep-sea, where bacteria have adapted to physical stresses such as extremes of temperature and pressure, exopolymers have been found that produce biochemically interesting EPS. The two environments highlighted in this review provide examples of reservoirs of microbial biodiversity that are relatively untapped. Several EPS produced by microbes from these extreme environments are showing biotechnological promise. By examining the chemical characteristics of these carbohydrate polymers, it is possible to begin to understand the ecological role of these natural products as well as to gain insight into their commercial potential.

2.11. Acknowledgements

Drs June Olley and Peter Nichols are thanks for their careful reading of this manuscript. The efforts of three anonymous referees for *Marine Biotechnology* are acknowledged as their comments significantly improved this review.

Chapter 3. Chemical characterization of exopolysaccharides from Antarctic marine bacteria

Chapter 3. Chemical characterization of exopolysaccharides from Antarctic marine bacteria

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The research within the original manuscript and this resultant Chapter was conducted by myself. Co-authors John Bowman and Jean Guezennec fulfilled supervisor roles. Sandrine Garon Lardière provided expertise in analyses of monosaccharides. John Gibson and Peter Nichols provided expertise in analyses of fatty acids including in the interpretation of mass spectral data.

3.1. ABSTRACT

Exopolysaccharides (EPS) may have an important role in the Antarctic marine environment, possibly acting as ligands for trace metal nutrients such as iron or providing cryoprotection for growth at low temperature and high salinity. Ten bacterial strains, isolated from Southern Ocean particulate material or from sea ice, were characterized. Whole cell fatty acid profiles and 16S rRNA gene sequences showed that the isolates included representatives of the genera *Pseudoalteromonas*, *Shewanella*, *Polaribacter* and *Flavobacterium* as well as one strain, which constituted a new bacterial genus in the family *Flavobacteriaceae*. The isolates are, therefore, members of the *"Gammaproteobacteria"* and *Cytophaga-Flexibacter-Bacteroides* (CFB), the taxonomic groups that have been shown to dominate polar sea ice and seawater microbial communities. EPS produced by Antarctic isolates were characterized. Chemical composition and molecular weight data revealed that these EPS were very diverse, even among six closely related *Pseudoalteromonas* isolates. Most of the EPS contained charged

uronic acid residues; several also contained sulfate groups. Some strains produced unusually large (molecular weight up to 5700 kDa) polymers, including one strain in which EPS synthesis is stimulated by low temperature. This study represents a first step in the understanding of the role a bacterial EPS in the Antarctic marine environment.

3.2. INTRODUCTION

Particulate aggregates are ubiquitous and abundant in the world's oceans (Fowler and Knauer 1986). As complex assemblages of zooplankton fecal pellets, phytoplankton and other material enriched in bacteria (Logan and Hunt 1987, Mueller-Niklas et al. 1994), marine aggregates are centers of high bacterial activity and are believed to have a significant role in the downward transport of carbon (Kiorboe 2001). Bacteria in marine aggregates are at an advantage compared to free living cells (Logan and Hunt 1987). Their proximity to other cells and surfaces provides opportunities for interaction and nutrient uptake. Exopolysaccharides (EPS) excreted by bacteria are among the polymeric substances that provide a network to hold these structures together (Flemming and Wingender 2001a). Microscopic and laboratory studies have shown that bacterially produced EPS have a major role in aggregate formation (Biddanda 1986, Heissenberger and Herndl 1994, Leppard 1995)

Bacteria contribute significantly to secondary production in sea ice communities and to the overall carbon cycle in the Antarctic environment. In thick pack ice, bacterial secondary production even exceeds primary production as the light supply to the bottom layers of ice is reduced (Grossmann and Dieckmann 1994). Bacteria are found in abundance in the bottom layers of the ice or in brine channels and are often attached to detrital particles or to living microalgal cells (Sullivan and Palmisano 1984, Archer et al. 1996, Delille and Rosier 1996). Delille and Rosier (1996) also suggest that the high numbers of particle associated bacteria found in sea ice may explain observations of underlying water enriched in bacterial biomass relative to the open ocean (Grossmann and Dieckmann 1994). More recently, studies of Arctic sea ice in winter showed that at temperatures as

low as –20°C, active bacteria were found in the brine channels and were particle associated (Junge et al. 2004). EPS may be providing a cryoprotectant role in these environments of high salinities and low temperature (Krembs et al. 2002). The same authors showed that high concentrations of EPS were found in brine channels and could have been produced by the abundant bacteria or diatom populations present.

EPS appears to be an integral component of particulate material and sea ice microbial communities, however very little is known about the chemical structures of EPS from cold adapted bacteria or bacteria from Antarctic waters. Basic knowledge of the chemical composition of EPS gives an insight into potential physico-chemical interactions that may take place within or between these polymers or between these polymers and cells or other substances in aquatic environments (Shin et al. 2001, Hirst et al. 2003). Within the polymer, the individual monosaccharides and functional groups covalently linked to them influence the weak cohesive forces that impact on the behavior of these polymers (Decho 1990). Chemical analysis of EPS from specific bacterial members of natural assemblages is difficult due to the low abundance of any one polymer and the complexity of tracing it back to its source (Christensen 1999). Therefore, Antarctic marine bacteria and the EPS they produced in pure liquid cultures were examined in this study as a first step in understanding the ecological significance of these abundant polymers in the Antarctic marine environment.

3.3. Materials and Methods

3.3.1. Isolation of bacteria

Samples were obtained during the November/December 2001 voyage of RSV *Aurora Australis* and included sea ice and seawater. The geographical position, the type of sample material and the size fraction from which bacterial isolates

Table 3.1. Source information for exopolysaccharide producing Antarctic marine bacterial isolates

Isolate	Position of sample site	Type of source material	Size fraction of source material	Treatment prior to culturing	Taxonomic grouping
CAM005	66° 38' 10"S, 141° 42' 76"E	melted fast ice	> 0.8 µm	filtering/enrichment	*Flavobacterium sp.*
CAM030	65° 32' 06"S, 143° 10' 16"E	plankton tow	> 20 µm	none	*Flavobacteriaceae*
CAM006	65° 32' 06"S, 143° 10' 16"E	plankton tow	> 20 µm	none	*Polaribacter sp.*
CAM003	63° 52' 41"S, 139° 41' 47"E	melted pack ice	> 0.8 µm	filtering/enrichment	*Pseudoaltermonas sp.*
CAM015	65° 03"S, 142° 31' 61"E	melted fast ice	< 0.8 µm	filtering/enrichment	*Pseudoaltermonas sp.*
CAM023	65° 32' 06"S, 143° 10' 16"E	plankton tow	> 20 µm	none	*Pseudoaltermonas sp.*
CAM025	63° 52' 04"S, 139° 41' 47"E	melted pack ice	< 0.8 µm	filtering/enrichment	*Pseudoaltermonas sp.*
CAM036	65° 32' 06"S, 143° 10' 16"E	plankton tow	> 20 µm	none	*Pseudoaltermonas sp.*
CAM064	65° 03"S, 142° 31' 61"E	melted fast ice	> 0.8 µm	filtering/enrichment	*Pseudoaltermonas sp.*
CAM090	66° 38' 10"S, 14142' 76"E	melted fast ice	> 0.8 µm	filtering/enrichment	*Shewanella sp.*

described in this study were obtained is shown in Table 3.1. Strains CAM006, CAM030, CAM023, CAM036 were isolated from particulate material from the Southern Ocean where the sea temperature was approximately 4°C and salinity was 3.5% (w/v). CAM005, CAM003, CAM015, CAM025, CAM064, CAM090 were obtained from melted sea ice. Pieces of sea ice (250-500 ml volume) from the bottom layer of ice floes (containing a visible basal algal band assemblage) were collected with a long handled sieve. Sea ice samples including interstitial seawater were combined with 500 ml sterile artificial seawater (2.2% w/v, Sigma) at 2°C. Aliquots were plated directly or the samples were stored at 2°C for two weeks. This storage temperature was preferable to the alternative (-20°C) for shipboard storage, as the latter would have caused additional freezing of the core and associated seawater, resulting in dramatically reduced viability of isolates.

Upon return to Hobart, aliquots of 200 ml of melted sea ice were passed through a Nucleopore 0.8 µm filter placed over a glass fiber filter (Schleicher & Schuell), which had been preheated to 450°C for one hour. The 0.8 µm filter or aliquots of 200 µl of the filtrate were placed in separate glass McCartney bottles containing 10 ml seawater nutrient broth (SNB, Bowman and Nichols 2002). These enrichment cultures containing medium and filters or medium and filtrate were incubated for 24 hrs at 2°C, then mixed and 200 µl aliquots were removed and spread onto SNAgar (SNB with 12 g l^{-1} agar added prior to sterilization) and SNAgar + Glucose (2% w/v). Agar plates were incubated at 4°C for four weeks.

Additional isolates were obtained from a plankton net (20 µm) trawled through the Southern Ocean. Aliquots of 20 µl of material from the cod end of the plankton net were spread onto SNAgar and SNAgar + Glucose (2% w/v) and the plates were incubated at 2°C.

After initial isolations, strains were subcultured onto marine agar (MA, 1 g yeast extract, Oxoid L21; 5 g bacteriological peptone, Oxoid L37; 32 g artificial sea salts, Sigma S9883; 15 g agar; 1000 ml distilled water) and MA supplemented with 3% (w/v) glucose (MA+Glu). A glucose solution was prepared and

autoclaved separately before being combined with MA. Strains were selected if they displayed a mucoid morphology when grown on MA+Glu. Approximately 300 isolates demonstrating a mucoid morphology were obtained initially from sea ice and marine particulate samples. The ten strains that showed the best growth on MA+Glu at 20°C with enhanced mucoid morphology were chosen for further analysis.

3.3.2. Characterization of bacterial isolates

3.3.2.1. 16S rRNA gene sequence analyses

The 16S rRNA genes of ten strains were amplified by PCR according to procedures described by (Bowman et al. 1996) using DNA primers 10F and 1519R. The PCR products were purified by Prep-A-Gene purification (Bio-Rad, CA, USA) and the concentration of purified DNA in each sample was measured using a Smart Spec 3000 (Bio-Rad, Regent Park, NSW, Australia). Sequences were obtained with a Beckman Coulter CEQ 2000 automated sequencer according to protocol specified by manufacturer (Beckman Coulter, Inc, Fullerton, CA, USA).

Sequences were manipulated and aligned using BioEdit v. 5.0.9 (Hall 1999). Sequences were compared to 16S rRNA genes available in the GenBank library by BLAST searching (Atschul et al. 1990) through the National Center for Biotechnology Information (U.S. National Institute of Health) internet site as described by Bowman et al. (1997c). Sequences were aligned to their closest related sequences determined from the BLAST searches. PHYLIP (version 3.57c) (Felsenstein 1993) was used to analyse the sequence data and sequence similarities with the maximum likelihood algorithm option were determined using DNADIST. Phylogenetic trees were constructed by the neighborliness method with the program NEIGHBOR. The sequence for *Escherichia coli* (J01695) was included as an outgroup. Partial sequences for the ten isolates were deposited into GenBank under accession numbers: AY243365, AY243366 and AY586522 to AY586529.

3.3.2.2. Whole-cell fatty acid analyses

Growth conditions were the same for all strains so that whole cell fatty acid profiles could be compared. The ten isolates were grown on MA at 12°C for four weeks. This incubation temperature was chosen as it approximates a median growth temperature for Antarctic sea ice bacteria (Bowman et al. 1997a). Whole cell fatty acids were extracted from cell material according to the MIDI protocol (Sasser 1990). Fatty acid methyl esters (FAME) were treated with N,O-bis-(trimethylsilyl)-trifluoroacetamide to convert hydroxy acids to their corresponding trimethylsilyl (TMSi) ethers for analysis by gas chromatography (GC) and GC-mass spectrometry. Double bond position and geometry of monounsaturated FAME for selected strains were determined after the formation of dimethyl-disulfide (DMDS) adducts prepared according to methods described previously (Nichols et al. 1986).

GC analyses were performed on a Hewlett Packard 5890A GC fitted with an HP-5 cross-linked methyl silicone fused capillary column (50 m x 0.32 mm i.d.) and flame ionization detector (FID, 310°C) and an HP 7673A auto sampler. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 50°C. After one minute, the oven temperature was raised to 150°C at 30°C min^{-1} then to 250°C at 2°C min^{-1} and finally to 300°C at 5°C min^{-1}. GC/MS analyses of the FAME were performed using a Finnigan GCQ Plus GC/MS system fitted with on-column injection set at 45°C. Samples were injected using an AS2000 auto sampler into a retention gap attached to a HP 5 Ultra 2, (50 m x 0.32 mm i.d., and 0.17 μm film thickness column using helium for the carrier gas. The chromatograms and mass spectra were analysed using Excalibur software. Peaks were identified by comparison to known and laboratory standards, the library included with the software, and by consideration of the mass spectra.

Fatty acids are designated by the total number of carbon atoms:number of double bonds, followed by the position of the double bond from the terminal (ω) end of the molecule. The suffixes c and t indicate *cis* and *trans* geometry and the prefixes

i and a indicate iso and anteiso branching. The position of hydroxyl group (OH) may occur on the first (α) or second (β) carbon from the carboxyl end of the molecule

3.3.3. EPS production

3.3.3.1. Growth of Antarctic isolates in batch cultures for EPS production

A McCartney bottle containing 20 ml marine broth supplemented with 3% (w/v) glucose (MB+Glu) was inoculated with approximately ten colonies of each strain from an agar plate (MA+Glu) inoculated ten days earlier and incubated at 20°C. The 20 ml cultures were shaken for 24 hr (200 rpm) at 20°C. These cultures were used to inoculate 200 ml of the same media and the resulting culture was shaken (200 rpm) and incubated at 20°C. After 48 hr, this broth was checked for contamination by subculturing onto a MA+Glu plate and a 10 ml aliquot was removed to measure pH. The remaining broth was used to inoculate 500 ml MB+Glu (pH 7) in a 2 l Schott bottle.

This 500 ml broth culture was bubbled with compressed air (138 kPa). Inlet and outlet air was filtered through a 0.2 μm Midisart filter (Sartorius Australia Pty. Ltd, VIC). Broth cultures were shaken (150 rpm) at 20°C for one week. Subculturing onto MA+Glu was used to check for contamination.

3.3.3.2. Isolation and purification of bacterial EPS

EPS were harvested according to procedures described previously (Mancuso Nichols et al. 2004). Briefly, culture broths were centrifuged at 30000 g for 2 hr at 4°C and the supernatants were pressure filtered through cellulose nitrate filters (Sartorius Australia Pty. Ltd., East Oakleigh, VIC). EPS were precipitated from the final filtrate after the addition of cold ethanol and the resulting precipitates were washed with 70% to 100% ethanol – water mixtures. The EPS were dried in a desiccator and stored at room temperature. To remove excess salts, the EPS were redissolved in distilled water and dialyzed (molecular weight cut off of 100 kDa, Spectra/Por, Spectrum Laboratories, Rancho Dominguez, CA, USA) against

distilled water for 2 days at room temperature (approx 25°C). Excess water was removed under vacuum before lyophilization. The EPS were stored at room temperature until analysis.

3.3.4. EPS Characterization

3.3.4.1. Colorimetric Analyses

Uronic acid content of the EPS was determined by the meta-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973, Filisetti-Cozzi and Carpita 1991). Protein content was determined by the Lowry protein assay (Lowry et al. 1951) with bovine serum albumin as the standard. The total neutral carbohydrate content was determined by the orcinol-sulfuric acid method modified by Rimington (1931).

3.3.4.2. FT-IR Spectroscopy

Pellets for infrared analysis were prepared by grinding a mixture of 2 mg polysaccharide with 200 mg dry KBr, followed by pressing the mixture into a 16 mm diameter mold. The Fourier transform-infrared (FT-IR) spectra were recorded on a Bruker Vector 22 instrument with a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} region. Approximate sulfate content was determined by FT-IR spectroscopy according to the method of Lijour et al. (1994).

3.3.4.3. Monosaccharide analyses

To a solution containing 250 µg total EPS, 50 µg erythritol was added as an internal standard. Samples for GC analyses were prepared in triplicate and freeze-dried. The polymer was hydrolysed by the addition of methanolic HCl (3N, Supelco, Bellefonte, PA, USA) and heating for 16 hr at 80°C (Kamerling et al. 1975, modified by Montreuil et al. 1986). The monosaccharides were converted to their TMSi derivatives by the addition of Bis-(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane / 99:1 (Supelco) and pyridine. The samples were held at room temperature for 2 hr, then dried under nitrogen and redissolved in dichloromethane prior to analyses.

3.3.4.4. Gas Chromatography

Analyses of the monosaccharides as TMSi derivatives were performed on a GC8000 (Fisons, Paris, France) GC fitted with an automatic injector, an FID (300°C) and a CP-Sil-5CB glass capillary column (0.32 mm x 60 m, Chrompac, Varian, Les Ulis, France). Hydrogen was the carrier gas. The GC oven was temperature programmed as follows: 50°C for 1 min then an increase of 20°C/min until 120°C, followed by a gradient of 2°C/min until 240°C.

3.3.4.5. Size exclusion chromatography

Molecular weights of EPS were determined by size exclusion chromatography (SEC). The eluent was 0.1 M $NaNO_3$ in water with 0.02% (w/v) sodium azide added. The analyses were carried out at room temperature. An isocratic pump (Spectra Physics P100, Thermo Separations, Bordeaux, France) with a flow rate of 1 ml/min and a TSK gel PW6000 column (Toyo Haas, Frankfurt, Germany) was used (60 cm x 0.7 cm) with pectin as a standard. For detection, a differential refractometer (Shodex RI71, Shodex, Tokyo, Japan) and a multiangle light scattering detector described elsewhere (Busnel et al. 1995) were employed. The polydispersity (I_p) is the ratio of the molecular weights based on the weight of the molecules (M_w) to the molecular weight based on the number of molecules (M_n), relative to the pectine standard ($M_w / M_n = I_p$).

3.4. Results

3.4.1. Isolation and characterization of bacteria

Several hundred isolates from sea ice and particulate material displayed mucoid morphology at 2°C on media with added glucose. Due to time constraints, the ten isolates that showed the best growth and enhanced mucoid morphology on MA+Glu, relative to MA at 20°C were selected for further characterization. All ten isolates were Gram-negative rods and showed growth in the temperature range of 4°C to 25°C. None of the strains was able to grow at 37°C. These isolates therefore, appear to be psychrotolerant.

According to 16S rRNA gene sequencing results, the ten isolates belonged to the order *Alteromonadaceae* (Figure 3.1) and the family *Flavobacteriaceae* (Figure 3.2). CAM003, CAM015, CAM023, CAM025, CAM036 and CAM064 were closely related and grouped in a sub-branch of the genus *Pseudoalteromonas* in which many Antarctic strains are found (Bowman 1998). The fatty acid profiles for these six *Pseudoalteromonas* isolates were consistent with the 16S rRNA gene identifications and are supported by other studies (Bozal et al. 1997, Bowman 1998, Table 3.2). Based on 16S rRNA gene sequence data, CAM090 was most similar to *Shewanella livingstonensis* (Figure 3.1). The whole cell fatty acid profile of CAM090 was very similar to that presented by Bozal et al. (2002) for *Shewanella livingstonensis* and included i13:0 and 16:1ω7c as the most abundant whole cell fatty acids. The whole cell fatty acid profiles for CAM003, CAM015, CAM023, CAM025, CAM036 and CAM064 showed many similarities to that of CAM090 (Table 3.2). As the genera *Pseudoalteromonas* and *Shewanella* belong to the order *Alteromonadaceae,* these seven isolates are therefore members of the class, "*Gammaproteobacteria*".

CAM006 was identified by 16S rRNA gene sequencing as being closely related to *Polaribacter irgensii* (Gosink et al. 1998) and CAM005 was related to *Flavobacterium frigidarium* (Humphry et al. 2001). Strain CAM030 was distant from validly described species within family *Flavobacteriaceae* and was most closely related to the species *Myroides odoratus* (Gherna and Woese 1992, Vancanneyt et al. 1996, Figure 3.2). As a group, CAM005, CAM006 and

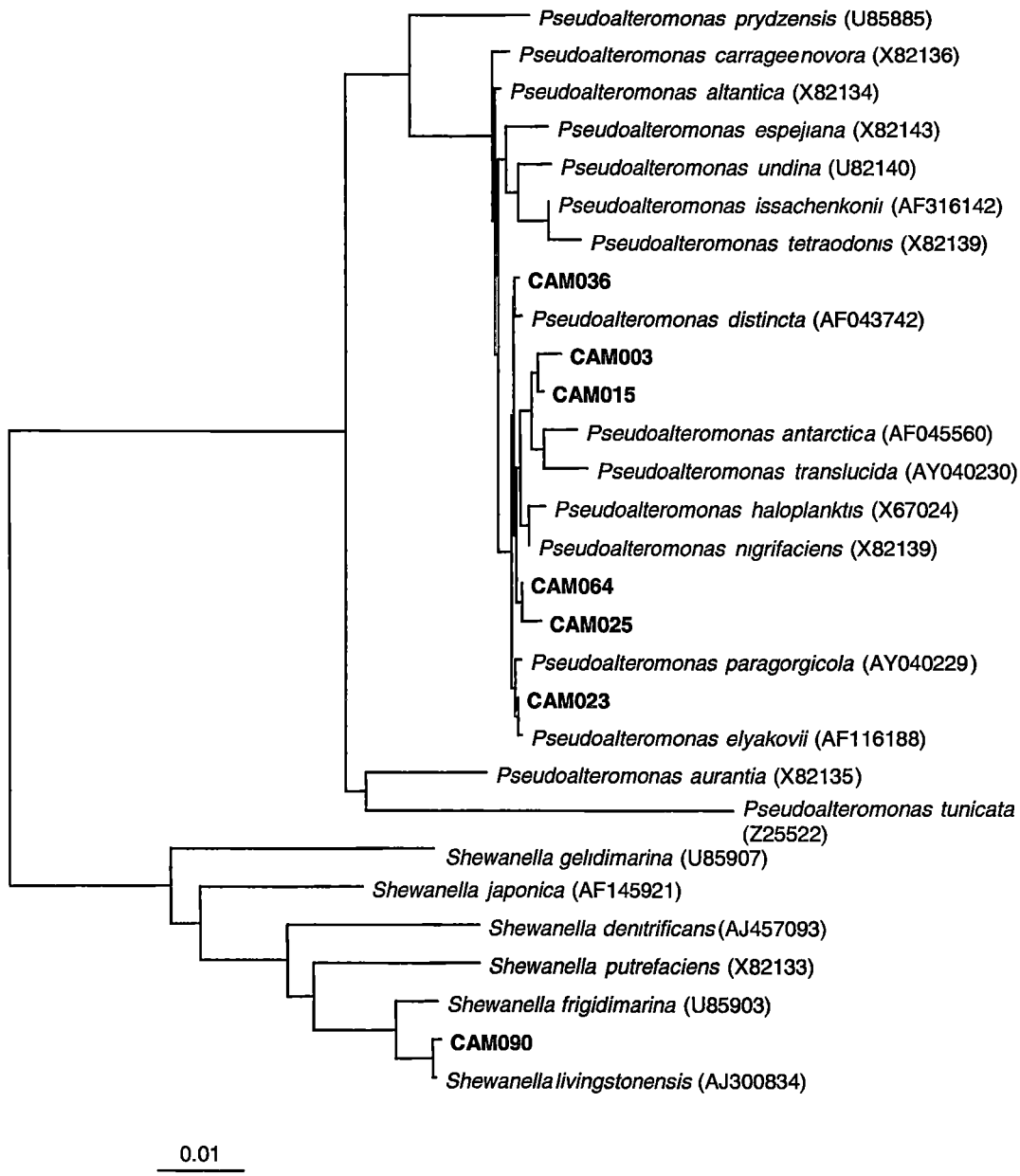

Figure 3.1. Phylogenetic relationship of Antarctic marine bacterial isolates (CAM036, CAM003, CAM015, CAM064, CAM025 and CAM090) in the order *Alteromonadaceae* based on 16S rRNA sequences. The tree was created using maximum-likelihood distanced clustered by the neighbor-joining method. Bar: 0.01 changes per mean nucleotide position. Numbers in parentheses are GenBank nucleotide accession numbers. Outgroup for the analysis was *Escherichia coli* (J01695).

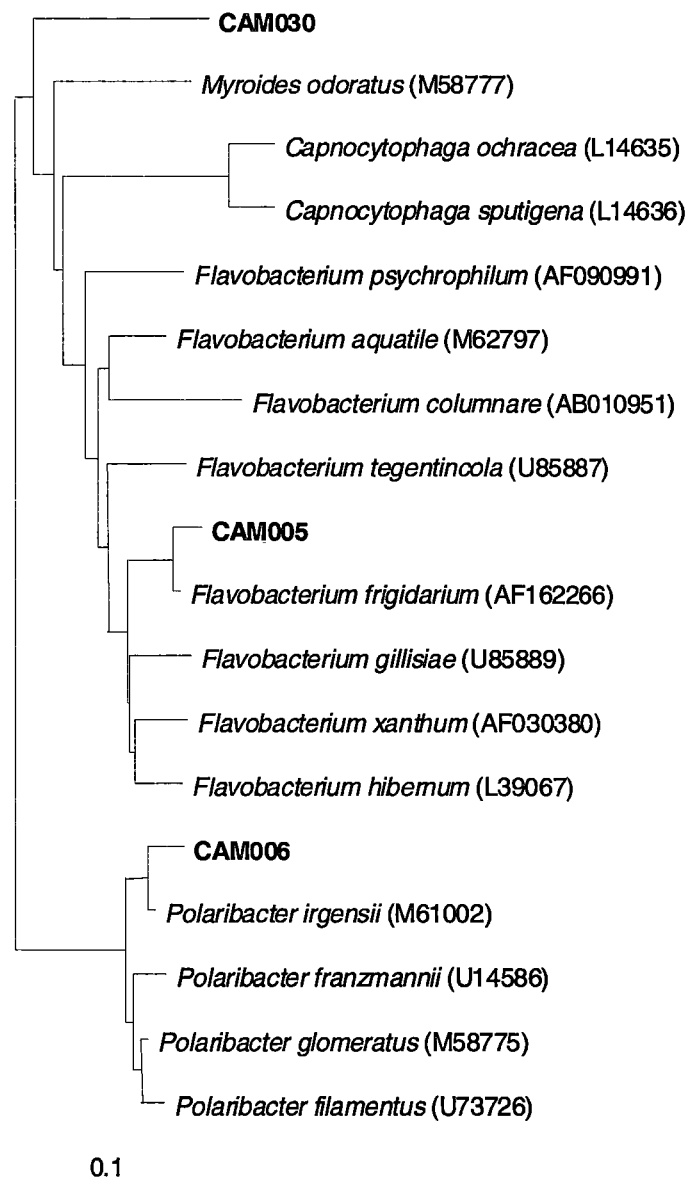

Figure 3.2. Phylogenetic relationship of Antarctic marine bacterial isolates (CAM030, CAM005 and CAM006) in the family *Flavobacteriaceae* based on 16S rRNA gene sequences. The tree was created using maximum-likelihood distanced clustered by the neighbor-joining method. Bar: 0.1 changes per mean nucleotide position. Numbers in parentheses are GenBank nucleotide accession numbers. Outgroup for the analysis was *Escherichia coli* (J01695).

Table 3.2. Whole cell fatty acids of ten Antarctic marine bacterial isolates reported as percentage of total fatty acids

	Isolates									
Fatty Acid	**CAM005**	**CAM030**	**CAM006**	**CAM003**	**CAM015**	**CAM023**	**CAM025**	**CAM036**	**CAM064**	**CAM090**
i13:0	1.0	tr*	3.0	3.6						18.9
13:0	tr	tr	1.6	3.1	tr	tr	tr	tr	3.4	4.0
β-OH i12:0				1.6	2.0	1.7	1 5	2.3	2.1	tr
14:0	tr	tr	tr	1.1	tr	tr	tr	tr	tr	2.1
β-OH 12:0				3.1	4.3	3.3	3.5	6.5	6.5	2.0
i15:1ω10c	9.3	21.9	13.7			2.4	1.9	2.1	3.5	
a15:1ω10c	1.7	7.9	tr							
i15:0	9.6	18.8	14.9							5.0
a15:0	3.9	4.0	1.1							
α-OH i13:0			2.4							
15:1ω8c				3.5	4.5	3.9	4.0	3.3	2.5	5.3
15:1ω6c			3.7							
α-OH i13:1	2.5	1.7								
15:1ω8t				1.7	tr	1.1	1.5	1.8	1.0	tr
15:1w6t	1.8	2.1								
15:0	3 2	7.3	4.0	4.6	4.2	3.7	4.7	3.3	3.7	3.2
β-OH 13:0	tr	tr	tr	tr	2.0	2.2	2.2	3.3	2.6	1.7
i16:1ω6c	2.9	tr	tr							
i16:0	4.0	tr		1.1	tr	1.2	tr	tr	tr	
16:1ω9c	1.4			1.0	tr	tr	tr	tr	tr	1.2
16:1ω7c	5.3	1.3	8.3	31.1	26.3	18.8	21.4	26.2	21.7	38.2
16:1ω7t			1.7	7.8	3.8	8.6	7.5	6.7	7.9	
β-OH 14:1									tr	3.5
16:0	1.5	1.2	tr	9.1	9.2	7.6	7.5	7.3	8.6	9.0
β-OH 14:0										2.0
β-OH i15:0	12.2	10.4	20.6							
α-OH i15:0		6.8	14.4							
α-OH a15:0	2.0	1.7	2.1							
17:1ω8c				20 5	26 9	29.2	29.3	24.2	21.0	10.2
17:1ω8t				3.8	4.4	6.5	6 8	4.1	4.1	2.2
17:0	tr			4.5	6.5	5.7	4 8	5.6	5.1	3.2
β-OH 15:0	1.5	3.8	3.1							
α-OH 15:0			1 4							
α-OH i16:0	11.4	4.4	4.4							
18:1ω9c	tr			tr	tr	tr	tr		tr	1.7
18:1ω7c	1.3	tr		5.4	5.8	4.2	3 5	3.3	6.3	8.6
β-OH 16:0	7.3	tr	tr							
β-OH i17:0	12.9	4.4	1.2							

^ listed in order of retention time
***tr : <1% total area**
Also present in trace amounts in the following strains:
CAM005: i14:0, a17:1, i17:1, β-OH 17:1; CAM030: a17:1, i17:1, β-OH i17:1, β-OH 17:1;
CAM006: i14:0, α-OH 13:0, β-OH i17:1; CAM003: i17:0, 18:0; CAM015: i17:0, 18:0; CAM023: i17:0, 18:0;
CAM064: 18:0; CAM090: i14:0, 18:0

CAM030 had whole cell fatty acid profiles typical of the *Cytophaga-Flexibacter-Bacteriodes* (CFB) phylum, and were dominated by branched chain fatty acids including: i15:1ω10c, i15:0 and β-OH i15:0. Differences were apparent between the whole cell fatty acid profiles of the three strains (Table 3.2). A study by Gosink et al. (1998) showed that β-OH i15:0 was the most abundant (38%) whole cell fatty acid in *Polaribacter irigensii*. We found β-OH i15:0 present at 20% of the total fatty acids and i15:0 (15%) as the second most abundant whole cell fatty acid in CAM006 and this was in agreement with the earlier findings. In a study by Bernardet et al. (1996) the most abundant whole cell fatty acids of *Flavobacterium* species were reported and are consistent with the whole cell fatty acid profile of *Flavobacterium odoratus* (since renamed as *Myroides odoratus*) and were identified as: i15:0 (39%) and β-OH i17:0 (9%). These were also abundant in CAM030: i15:0 (19%), β-OH i17:0 (4%). There were many minor whole cell fatty acids common between the strains, however, one obvious difference was the presence in large amounts of i15:1ω10c in CAM030 (22%) and in the other *Flavobacterium* species reported in this study but the absence of this fatty acid in *Myroides odoratus*. As 16S rRNA gene sequencing results showed that CAM030 was more closely related to *Myroides odoratus* than to any of the other genera in the *Flavobacteriaceae* family, further work is necessary to identify this Antarctic marine isolate. Phylogenetically these three isolates, as members of family *Flavobacteriaceae*, are also part of the larger CFB phylum.

3.4.2. Chemical characterization of EPS

Figure 3.3 shows the approximate contribution of neutral sugars, uronic acids, amino sugars, sulfates and proteins to the EPS produced by each strain. Data are reported as percentages of the total EPS on a weight basis. Neutral sugars accounted for nearly half of all ten EPS. Uronic acids made up the second most abundant fraction of EPS from CAM030, CAM023, CAM025, CAM036, CAM064 and CAM090. Protein was a major component of EPS from CAM005,

CAM006 and CAM015. The protein content in EPS from CAM005 and CAM006 was 40% and 50% respectively. Purification of these EPS included high-speed centrifugation, followed by pressure filtration, dialysis (100 kDa cut-off) and ethanol precipitation. These steps were expected to have removed cellular material and unbound proteins, which would have otherwise contributed to elevated protein levels. The protein in these EPS may be bound to the polysaccharide and further analyses are required to better understand these structures. FT-IR analyses showed EPS from CAM003 to have approximately 15% sulfate, while EPS of strains CAM025, CAM036 and CAM064 contained lower percentages of sulfate. Amino sugars were present in EPS from several strains as determined by GC analysis (Figure 3.3).

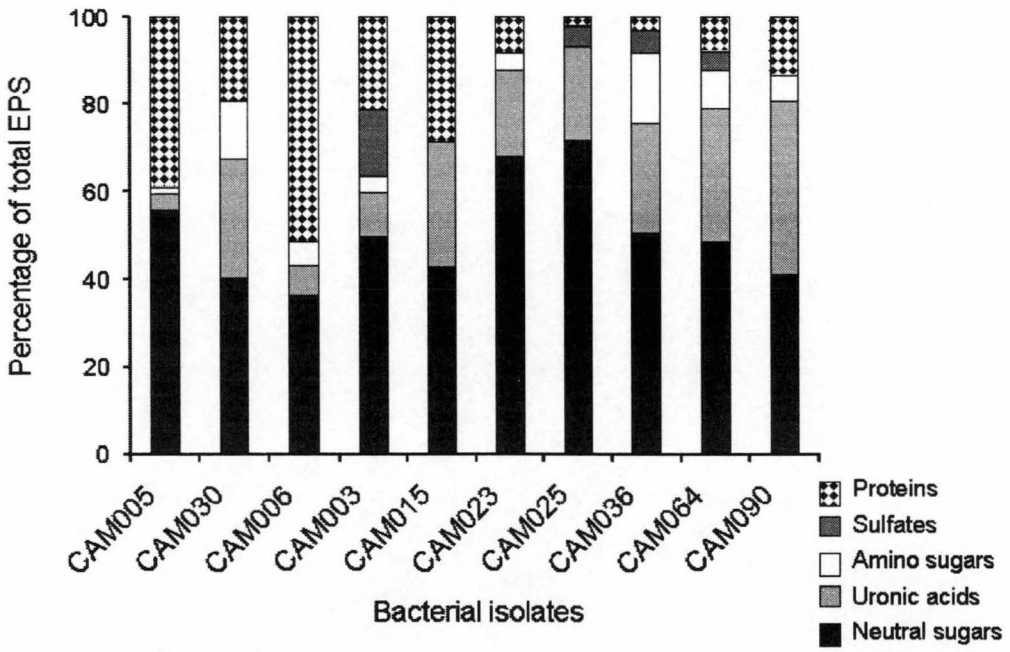

Figure 3.3. Crude chemical composition of exopolysaccharides produced by Antarctic marine bacteria. Neutral sugars, uronic acids, proteins were determine by colorimetric analysis. Sulfate content was estimated from FT-IR spectra. Amino sugar content was determined by gas chromatography.

The abundances of monosaccharide components in EPS produced by each isolate were presented as percentage of total monosaccharides (Table 3.3). Mannose represented the most abundant neutral sugar in the EPS, especially in the EPS of strains CAM003, CAM005, CAM030, CAM006, CAM064 and CAM090. *Pseudoalteromonas* sp. CAM003 contained high percentages of both fucose and mannose whereas closely related strains CAM023 and CAM025, included glucose as the most abundant neutral sugar. *Pseudoalteromonas* sp. CAM036 contained mannose and glucose as well as galacturonic acid in nearly equal amounts, on a percentage basis. N-acetyl galactosamine and N-acetyl glucosamine, were present in high amounts in CAM030 (approximately 10%). N-acetyl galactosamine was also present in CAM036 and CAM064 at 14 and 11% respectively (Table 3.3).

Average molecular weights of EPS are presented in Table 3.4. Polydispersity gives an indication of the molecular weight distribution or range of size homogeneity within each polymer. As polydispersity increases, there is a larger difference between the sizes of the largest molecules and the smallest molecules. All polymers in this study had a polydispersity below 2.5 based on the molecular weight measurements. This data indicates that for each EPS analyzed, only one polymer was present and this polymer was relatively homogeneous with respect to molecular weight.

The EPS produced by strains CAM003, CAM005, CAM006, CAM015, CAM023, CAM025 and CAM036 had molecular weights greater than 1700 kDa. CAM030, CAM064 and CAM090 produced EPS with molecular weights less than 200 kDa. Molecular weight measurements also indicated a wide range in the average molecular weight of EPS produced by the Antarctic marine isolates examined in this study. Large differences were apparent between the EPS produced by the closely related strains CAM025 and CAM064, with CAM025 producing the EPS with the highest molecular weight (5700 kDa) and CAM064 producing one of the smallest (100 kDa).

Table 3.3 EPS monosaccharides from ten Antarctic marine bacterial isolates reported at percentage of total sugars

	Isolates									
Monosaccharides	CAM005	CAM030	CAM006	CAM003	CAM015	CAM023	CAM025	CAM036	CAM064	CAM090
Ara*	5	6	2	4	10	12	3	3	4	13
Rib				2			1			
Rha		1		6	6		5			2
Fuc			11	29			1			
Xyl		2			1					2
Man	74	48	33	40	36	2	1	24	64	41
Gal	3	4	38		4	1	5	1	4	5
Glc	8	9	4	16	38	75	52	26	8	10
Gal A		2				5	30	30		
Glc A	8	10	6	1	3	3			6	20
N-A Gal		10	1	1	3	2	1	14	11	7
N-A Glc	1	8	4	1				1	2	

*Ara indicates arabinose; Rib, ribose; Rha, rhamnose; Fuc, fucose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; Gal A, galacuronic acid; Glc A, glucuronic acid; N-A Gal, N-acetyl-galactosamine and N-A Glc, N-acetyl-glucosamine.

Table 3.4. Molecular weights of exopolysaccharides produced by Antarctic marine bacteria determined by Size Exclusion Chromatography

Isolate	Molecular weight (kDa)	I_p (polydispersity)
CAM005	1805	1.249
CAM030	187	1.891
CAM006	2100	1.224
CAM003	1800	2.075
CAM015	2800	1.143
CAM023	1800	1.381
CAM025	5700	2.496
CAM036	1700	1.616
CAM064	100	1.172
CAM090	81	1.326

3.5. Discussion

3.5.1. Characterization of bacteria

Studies of the diversity of polar sea ice and seawater microbial communities have shown that the phyla "*Gammaproteobacteria*" and CFB are the dominant taxonomic groupings generally found in this environment as determined by cultivation dependent and independent techniques (Bowman et al. 1997c, Simon et al. 1999, Staley and Gosink 1999, Brinkmeyer et al. 2003). The ten Antarctic marine bacterial isolates examined in this study belong to these phylogenetic groups as demonstrated by results from 16S rRNA gene sequencing and whole cell fatty acid analysis. Our data also indicated that six of the ten strains investigated were in a clade within the genus *Pseudoalteromonas* particularly common in the Southern Ocean, especially in sea ice (Bowman 1998).

3.5.2. Characterization of EPS

The EPS produced by the ten Antarctic strains were diverse with respect to crude chemical (Figure 3.3) and monosaccharide (Table 3.3) composition. In a study of *Pseudomonas antarctica*, NF_3 (Bozal et al. 1994, Bozal et al. 1997), crude chemical analysis of exopolymers produced by this strain showed a high protein content, while individual monosaccharide data were not presented. Capsular material, generally high in proteins (Kenne and Lindberg 1983), was included in the fraction analysed in this earlier study. As our study examines EPS excreted into the media, comparison of these two data sets is not possible. A study by Corsaro et al. (2004) examined the exopolymers produced by another Antarctic marine isolate, *Pseudomonas haloplanktis* TAC 125, and showed the polysaccharide component to consist of mannose with traces of glucose. Since the fraction examined in this study also included the LPS layer, it is not possible to compare these results with those of our study. Excreted EPS produced by non-marine strains was analysed in a study by Ford et al. (1991). Uronic acids made up less that 1% of the total carbohydrates. This is significantly lower than the uronic content of the EPS examined in the current study.

The EPS produced by several deep-sea hydrothermal vent bacterial isolates have been well characterized (Rougeaux et al. 1996), including two from genus *Pseudoalteromnas*. Despite the strains belonging to two different species, the EPS produced by these deep-sea isolates were very similar with respect to crude chemical and monosaccharide composition. These results contrast with those presented in a previous study (Mancuso Nichols et al. 2004) and this one, in which EPS produced by closely related strains in the genus *Pseudoalteromonas* vary substantially. Sulfate content was noted as the only structural difference between the polymers in the two hydrothermal vent strains and sulfate content may have had an influence on the intrinsic viscosity of these polymers since it also varied. Another study found two closely related hydrothermal vent bacteria from different subspecies of *Alteromonas macleodii*, and isolated from different sites, produced very different EPS under the same growth conditions (Cambon-Bonavita et al.

2002). These EPS show a high metal binding capacity (Loaec et al. 1998). This quality has been attributed to chemical characteristic also present in the EPS produced by the Antarctic marine isolates examined in this study, including carboxyl groups present in uronic acids, amides present in amino sugars, sulfates and to a lesser extent to hydroxyl groups, which are abundant in all monosaccharides. These EPS are thought to aid in attaching bacteria to the hydrothermal chimney as well as lowering the concentrations of toxic heavy metals in the microenvironment.

Monosaccharide analyses of the ten EPS examined in this study showed that pentoses (ribose and xylose), hexoses (glucose, galactose, mannose, rhamnose and fucose), amino sugars (N-acetyl glucosamine, N-acetyl galactosamine) and uronic acids (glucuronic acid and galacturonic acid) were present, with glucose, galactose or mannose being the most abundant monosaccharides. These sugars are typically found in bacterial EPS (Kenne and Lindberg 1983). A survey of EPS produced by pure cultures of seawater isolates by Kennedy and Sutherland (1987) shows that this is also the case for EPS produced by marine bacteria. Arabinose was present, to varying degrees, in all EPS examined in this study and xylose was present as a minor component in CAM030, CAM090 and CAM090. Arabinose and xylose are not commonly found in bacterial EPS (Kenne and Lindberg 1983). The significance of these finding requires further investigation.

3.5.3. The ecological role of EPS

In both sea ice and marine particulate material, bacteria are seen as an integral part of the trophodynamics and are credited with a critical importance in the organic carbon cycle (Grossmann and Dieckmann 1994, Azam 1998). Bacterial polysaccharides form the fibrillar frame work, act as glue in the ultrastructure and provide the structural network for microbial associations within marine aggregates (Flemming and Wingender 2001a, Biddanda 1986, Heissenberger et al. 1996, Decho and Herndl 1995). Studies of sea ice microbial communities also have found bacteria to be strongly particle associated (Sullivan and Palmisano 1984, Grossi et al. 1984). More recent results showed that microbial EPS was produced

in high concentrations and it was suggested that it played an important role in cryoprotection (Krembs et al. 2002, Junge et al. 2004).

Exopolymers produced by marine bacteria generally contain 20-50% of the polysaccharide as uronic acid (Kennedy and Sutherland 1987). Uronic acids contain an acidic carboxyl group that is ionisable at seawater pH. This contributes a negative charge to the overall polymer (Decho 1990). Sulfate was thought to occur only in polysaccharides produced by Archaea and Cyanobacteria until recently, and its presence in polymers produced by prokaryotes is seen as very uncommon (Arias et al. 2003). When sulfate is present as a functional group, it also contributes to the anionic quality of these EPS in seawater (Leppard et al. 1996). The overall negative charge gives the molecule a 'sticky' quality. This 'stickiness' is important in terms of the affinity of these EPS for binding to cations such as dissolved metals (Brown and Lester 1982). Based on the data presented, the EPS from our isolates, which contain uronic acids as well as sulfates, would be in a highly polyanionic state in the marine environment.

The availability of iron (Fe^{+3}) as a trace metal is of critical importance in the Southern Ocean where it is known to limit primary production (Scharek et al. 1997). As much as 99% of dissolved iron in the ocean is bound to organic ligands (Rue and Bruland 1995). Our results indicate that several of the EPS produced by Antarctic bacterial isolates derived from Southern Ocean particulate material included uronic acids and/or sulfates (CAM030, CAM006, CAM23 and CAM036, Figure 3). These EPS may be acting as ligands for cations such as iron and other trace metals in the Southern Ocean environment. A study of Arctic sea ice demonstrated that photosynthesis rates by phytoplankton from under the ice were stimulated to similar levels by sea ice extracts as they were by the chelator, ethylenediamine tetra-acetic acid and trace metals (Apollonio et al. 2002). From those results, the authors suggested that a natural 'conditioning agent' is produced within the bottom-ice algal layer that enhances phytoplankton growth. Sea ice bacterial communities and high amounts of EPS are concentrated in these layers (Krembs and Engel 2001, Krembs et al. 2002).

It is not clear whether dissolved iron is a limiting nutrient for primary production in sea ice microbial communities. Isolates derived from pack ice (CAM003 and CAM025) or from fast ice (CAM005, CAM064 and CAM090) produced EPS containing uronic acids and/or sulfates. These EPS would be negatively charged in the marine environment and could possibly have some role in enhancing primary production. The overall significance of this finding requires further investigation

EPS produced by sea ice isolates CAM003, CAM005 and CAM025 were shown, by molecular weight analysis to be 1800, 1800 and 5700 kDa, respectively. Most exopolysaccharides produced by marine bacteria have a molecular weight in the 100-300 kDa range (Decho 1990), therefore the EPS produced in liquid culture by three sea ice isolates examined in this study are between 5 and 50 times larger than average. The structure and properties of EPS are influenced by the length of the polymer chain, that is, the molecular weight (Christensen 1999). As the length of the polymer increases, there is a greater opportunity for complex entanglement of the chains and intramolecular associations, and these contribute to the tertiary structure and physical behavior of the polymer (Sutherland 1994). A fungal strain, *Phoma herbarum,* isolated from Antarctic soil produced a homosaccharide of glucose with a molecular weight of 7400 kDa (Selbmann et al. 2002). The authors of this study suggested the fungal EPS could provide a cryoprotective role in the harsh Antarctic environment where the availability of liquid water and temperatures are extremely low. Similarly, the freezing processes in sea ice result in brine channels where temperature is very low and salinity is high due to brine. Strain CAM025 was observed to produce 30 times as much EPS at -2 and 10°C compared to 20°C (Mancuso Nichols et al. 2004). Whether this increased EPS production at low temperature is a cold adaptation mechanism for this strain, requires further investigation. Bacterial EPS production in brine channels and perhaps other cold, high brine ecosystems may provide a barrier against the environmental extremes experienced by the bacterial cell by modifying water properties near the cell.

An exopolysaccharide, known as muran, is produced by the halophilic bacterium, *Halomonas maura* (Arias et al. 2003). When this strain was grown in media containing high salt concentration (2.5%, w/v), it produces a high molecular weight (4500 kDa) EPS that contained glucose, mannose and galactose as well as high amounts of glucuronic acid (8%, w/w) and sulfates (6.5%, w/w). This polysaccharide was able to bind a range of heavy metal cations. The authors also noted the stability of muran under different stress conditions including high salt concentrations and during freezing/thawing. There are similarities between muran and several of the EPS produced by our Antarctic marine isolates in terms of chemical composition. Further work is necessary to more accurately define the structure of the Antarctic marine bacterial EPS and to relate these findings to the function of these molecules in the natural environment. As yet, it is unclear how these polysaccharides may be acting mechanistically as organic ligands, protectants against low temperature or high salinity, or whether the size of these EPS is related to their ecological role.

3.5.4. Biotechnological potential

Polysaccharides with biotechnological potential have been derived from bacteria isolated from extreme environments such as hydrothermal vents and hypersaline habitats as mentioned above. Because of their physico-chemical properties, which include stabilizing, thickening, gelling, coagulating, adhesion and water retention, bacterial EPS have been used in many commercial applications for example in the pharmaceutical, food and environmental industries (Guezennec 2000, Flemming and Wingender 2001b, Sutherland 1998). EPS produced by Antarctic bacteria examined in the current study appear to be diverse with respect to chemical composition. These EPS also share chemical characteristics such as high uronic acid content, sulfate content and molecular weight found in polysaccharides that have been exploited commercially. A better understanding of the chemical structure of these complex molecules is necessary for the discovery of potential biotechnological uses as well as their role in the Antarctic marine environment.

3.6. Conclusions

Bacteria living in Antarctic sea ice and particulate material in the Southern Ocean experience extreme ecological pressures. EPS is produced by many marine bacteria isolated from this environment. This study is a first step towards understanding the role of these substances in the Antarctic marine environment. A set of ten isolates were found to belong to the "*Gammaproteobacteria*" and CFBs, which are dominant taxonomic groups in this environment. Study of the chemical composition of EPS produced by these isolates shows that there are distinct differences in terms of crude chemical composition, size and monosaccharide content, even for those EPS produced by closely related strains. Whether the specific ecological role for these bacterial EPS includes cryoprotection in the high salinity, low temperature brine channels in sea ice, or trace metal binding in marine aggregates remains to be elucidated. Increased knowledge of the role of Antarctic bacterial EPS will also provide insight into possible commercial uses for these novel polymers.

3.7. Acknowledgements

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Chapter 4. Production of exopolysaccharides by Antarctic marine bacterial isolates

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The research within the original manuscript and this resultant Chapter was conducted by myself. Co-authors John Bowman and Jean Guezennec fulfilled supervisory roles. Sandrine Garon provided expertise in analyses of monosaccharides and Gerard Raguénès provided assistance in the microbiological laboratory at IFREMER Centre de Brest, France.

4.1. SUMMARY

Aims: This study was undertaken to examine and characterize Antarctic marine bacterial isolates and the exopolysaccharides (EPS) they produce in laboratory culture.

Methods and Results: Two EPS-producing bacterial strains CAM025 and CAM036 were isolated from particulate material sampled from seawater and sea-ice in the Southern Ocean. Analyses of 16S rDNA sequences place these isolates in the genus *Pseudoalteromonas*. In batch culture, both strains produced EPS. The yield of EPS produced by CAM025 was 30 fold higher at –2°C and 10°C than at 20°C. Crude chemical analyses show that these EPS were composed primarily of neutral sugars and uronic acids with sulfates. Gas chromatographic analysis of monosaccharides confirmed these gross compositional findings and molar ratios of monosaccharides revealed differences between the two EPS.

Conclusions: The EPS produced by Antarctic bacterial isolates examined in this study appear to be polyanionic and, therefore, 'sticky' with respect to cations such as trace metals.

Significance and Impact of Study: Since the availability of iron as a trace metal is of critical importance in the Southern Ocean where it is know to limit primary production, the role of these bacterial EPS in the Antarctic marine environment has important ecological implications.

4.2. INTRODUCTION

The production of exopolysaccharides (EPS) by bacteria in natural systems has been described as a strategy for growth (Costerton 1999). Studies of bacteria growing in aquatic systems, such as marine sediments, aggregates and detrital particles, show that nearly all the cells are surrounded by EPS (Decho 1990, Costerton 1999) and many of these cells are enclosed with adherent biofilms (White 1986).

In the oceans, EPS exuded by phytoplankton and bacteria coalesce to form transparent exopolymer particles (TEP), which range in size from microns to hundreds of microns (Sullivan and Palmisano 1984, Passow and Alldredge 1994). The aggregation of TEP, phytoplankton, bacteria, faecal pellets, zooplankton and other organic debris form larger particles (> 0.5 mm in diameter), which are known as marine snow (Fowler and Knauer 1986). Marine snow has been shown to include highly concentrated and diverse microbial communities Rath et al. (1998) engaged in photosynthesis, microbial decomposition (Biddanda 1988) and remineralization of carbon at elevated levels relative to the surrounding sea water (Smith et al. 1992). Marine snow particles therefore make a significant contribution to carbon cycle in the euphotic zone and to the 'biological pump', which transports fixed carbon to deep waters (Alldredge 2000).

Microbial communities have been found associated with deep-sea hydrothermal vents. These ecosystems are characterized by extremely high temperatures and

pressures as well as high concentrations of toxic elements (*e.g.* sulfides and heavy metals). EPS-producing thermophilic and mesophilic strains have been isolated from vent environments. Several bacterial exopolymers were found to be novel with significant biotechnological potential (Guezennec et al. 1994).

In the Antarctic marine environment, annual sea-ice is a microhabitat for a complex community of marine bacteria often in close association phytoplankton. These assemblages are essential components of carbon and energy transfers in the Southern Ocean (Sullivan and Palmisano 1984). Abundant bacterial populations have been found in thick annual pack ice with psychrophilic bacteria being particularly common in samples of brown ice and pore waters (Delille 1992). Bacterially produced EPS may provide a means by which bacteria can adhere to the microalgal cells (Sullivan and Palmisano 1984). During ice formation microalgal cells are scavenged by sea-ice crystals floating up to the sea surface (Gleitz and Thomas 1993) and bacteria attached to algal cells may be incorporated into new ice in conjunction with some algal species (Grossmann and Dieckmann 1994).

Studies of both the Arctic (Krembs and Engel 2001) and Antarctic (Sullivan and Palmisano 1984) sea-ice communities suggest that exopolymer production by both phytoplankton and bacteria make a significant contribution to organic carbon in the sea-ice and ice-water interface. Sea-ice bacteria maintained in laboratory culture are reported to secrete copious amounts of mucous (Helmke and Weyland 1995). Little is known about EPS produced by Antarctic marine bacteria or about its role in this extreme environment. In this study, bacterial strains were isolated from the Antarctic marine environment. Two of these isolates and the EPS they produce were examined and results are presented.

4.3. Materials and Methods

4.3.1. Isolation of bacteria

Samples were obtained during the November/December 2001 voyage of RSV *Aurora Australis* (approximate location: 63°52'04"S, 139°41'47"E). Sea-ice

pieces were collected with a long handled sieve. Small chunks of approximately 250 ml to 500 ml of bottom ice (distinguished by algal pigment coloration in bottom 1 cm) were melted at 2°C in 500 ml artificial seawater (2.2% w/v, Sigma) and stored at 2°C for two weeks. Upon return to Hobart, aliquots of 200 ml of this liquid were passed through a Nucleopore 0.8 µm filters placed over a glass fibre filter (Schleicher & Schuell), which had been preheated to 450°C for one hour. The 0.8 µm filter was placed in a glass McCartney bottle containing 10 ml seawater nutrient broth (SNB) described previously (Bowman and Nichols 2002). These enrichment cultures containing media and filters were incubated for 24 hrs at 2°C, then mixed and 200 µl aliquots were removed and spread on SNAgar (SNB with 12 g/L agar added prior to sterilization) and SNAgar + Glucose (2% w/v). Agar plates were incubated at 4°C for four weeks. Additional isolates were obtained from a plankton net (20 µm) trawled through the Southern Ocean (approximate location: 65°32'06"S, 143°10'16"E). Aliquots of 20 µl of material from the cod end of the plankton net were spread onto SNAgar and SNAgar + Glucose (2% w/v) and the plates were incubated at 2°C.

After initial isolations, both strains were subcultured onto marine agar (MA, 1 g yeast extract, Oxoid L21; 5 g bacteriological peptone, Oxoid L37; 32 g artificial sea salts, Sigma S9883; 15 g agar; 1000 ml distilled water) and MA supplemented with 3% (w/v) glucose (MA+Glu). A glucose solution was prepared and autoclaved separately before being combined with MA. Strains were selected if they displayed a mucoid morphology when grown on MA+Glu

4.3.2. Characterization of bacterial isolates

4.3.2.1. 16S rDNA sequence analyses

The 16S rDNA genes of CAM025 and CAM036 were amplified by PCR according to procedures described by (Bowman et al. 1996) using DNA primers 10F and 1519R. The PCR products were purified by Prep-A-Gene purification (Bio-Rad, CA, USA) and the concentration of purified DNA in each sample was measured using a Smart Spec 3000 (Bio-Rad, Regent Park, NSW, Australia). The

16S rDNA sequences were obtained with a Beckman Coulter CEQ 2000 automated sequencer for electrophoresis and data collection after preparation according to protocol specified by manufacturer (Beckman Coulter, Inc, Fullerton, CA, USA).

Sequences were manipulated and aligned using BioEdit v. 5.0.9 (Hall 1999). Sequences were compared to 16S rDNA genes available in the GenBank library by BLAST searching (Atschul et al. 1990) through the National Center for Biotechnology Information (U.S. National Institute of Health) Internet site as described by Bowman et al. 1997c). Sequences were aligned to their closest related sequences determined from the BLAST searches. PHYLIP (version 3.57c) (Felsenstein 1993) was used to analyze the sequence data and sequence similarities with the maximum likelihood algorithm option were determined using DNADIST. Phylogenetic trees were constructed by the neighborliness method with the program NEIGHBOR. The sequence for *Escherichia coli* (J01695) was included as an outgroup. Partial sequences for these two isolates were deposited into GenBank and assigned the following numbers: AY243365 (CAM025) and AY243366 (CAM036).

4.3.2.2. Whole-cell fatty acid analyses

Growth temperatures may affect whole cell fatty acid profiles, therefore similar incubation temperatures to those used in other studies (Bozal et al. 1997, Bowman 1998) were used in this study so that whole cell fatty acid profiles could be compared. Isolates CAM025 and CAM036 were grown on MA at 12°C for four weeks. Whole cell fatty acids were extracted from cell material according the MIDI protocol (Sasser 1990). Fatty acid methyl esters (FAME) were treated with N,O-bis-(trimethylsilyl)-trifluoroacetamide to convert hydroxy acids to their corresponding trimethylsilyl (TMSi) ethers for analysis by gas chromatography (GC) and GC-mass spectrometry. Double bond position and geometry of monounsaturated FAME were determined after the formation of dimethyl-disulfide (DMDS) adducts prepared according to methods described previously

(Nichols et al. 1986). Determination of the *cis* and *trans* geometry in the original monounsaturated FAME was also possible (Skerratt et al. 1991).

GC analyses were performed on a Hewlett Packard 5890A GC fitted with an HP-5 cross-linked methyl silicone fused capillary column (50 m x 0.32 mm i.d.) and flame ionization detector (FID) and an HP 7673A auto sampler. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 50°C. After one minute, the oven temperature was raised to 150°C at 30°C/min then to 250°C at 2°C/min and finally to 300°C at 5°C/min. GC/MS analysis of the FAME was performed using a Finnigan GCQ Plus GC/MS System fitted with on-column injection set at 45°C. Samples were injected using an AS2000 auto sampler into a retention gap attached to a HP 5 Ultra 2, (50 m x 0.32 mm i.d., and 0.17 μm film thickness column using helium for the carrier gas. The chromatograms and mass spectra were manipulated using Excalibur software. Peaks were identified by comparison to known standards, the library included with the software, and by consideration of the mass spectra.

Fatty acids are designated by the total number of carbon atoms:number of double bonds, followed by the position of the double bond from the terminal (ω) end of the molecule. The suffixes c and t indicate *cis* and *trans* geometry; the prefixes I and a refer to iso and anteiso branching.

4.3.3. EPS production and characterization analysis

4.3.3.1. Growth of CAM025 and CAM036 in batch cultures for EPS production

A McCartney bottle containing 20 ml Marine broth supplemented with 3% (w/v) glucose (MB+Glu) was inoculated with approximately ten colonies of either CAM025 or CAM036 from an agar plate (MA+Glu) inoculated ten days earlier and incubated at 20°C. This incubation temperature was chosen as it approximates the optimum growth temperature measured for other *Pseudoalteromonas* strains isolated from the same environment (Bowman 1998). The 20 ml culture was shaken for 24 hr (200 rpm) at 20°C. This 20 ml culture was used to inoculate 200

ml of the same media and the resulting culture was shaken (200 rpm) and incubated at 20°C. After 48 hr, the purity of this culture was checked by subculturing onto a MA+Glu plate and a 10 ml aliquot was removed to measure pH. The remaining broth was used to inoculate 500 ml MB+Glu (pH 7) in a 2 L Schott bottle.

This 500 ml broth culture was bubbled with compressed air (20 lb/in^2). Inlet and outlet air was filtered through a 0.2 µm Midisart filter (Sartorius Australia Pty. Ltd, VIC). Broth cultures were shaken (150 rpm) at 20°C for one week. Purity of this broth was checked by subculturing onto MA+Glu.

The CAM025 isolate was also grown in duplicate 250 ml MB+Glu (pH 8) broth cultures at -2°C, 10°C and 20°C. The baffled flasks were incubated in oscillating water baths (Ratek Pty Ltd, Australia) fitted with refrigeration units, which cooled and circulated antifreeze liquid at the desired temperature. The batch cultures at –2°C, 10°C were harvested after 2 weeks incubation. The cultures at 20°C were harvested after 1 week. The final pH was measured for each culture at time of harvest.

4.3.3.2. Isolation and purification of CAM025 and CAM036 EPS

Culture broth was centrifuged at 30000 g for 2 hr at 4°C. The cell pellets were freeze-dried and weighed. The supernatants were pressure filtered successively through cellulose nitrate filters with the following pore sizes: 8.0 µm, 3.0 µm, 1.2 µm, 0.8 µm and 0.45 µm (Sartorius Australia Pty. Ltd., VIC). EPS were precipitated from the final filtrate after the addition of cold ethanol (filtrate 60 ml / ethanol 40 ml) and the solution was chilled to 2°C over night. The resulting precipitate was recovered by vacuum filtration through sintered glass. An additional 100 ml cold ethanol was added to the filtrate and the solution was placed at –20°C overnight. The precipitate was recovered as above. The precipitates were washed with 70% to 100% ethanol – water mixtures. After washing with ethanol, the EPS were combined and dried in a desiccator and stored

at room temperature. To remove excess salts, the EPS were redissolved in distilled water and dialyzed (molecular weight cut off of 100 kDa, Spectra/Por, Spectrum Laboratories, CA, USA) against distilled water for 2 days at room temperature (approx 25°C). Excess water was removed under vacuum before lyophilization. The EPS were stored at room temperature until analysis.

4.3.4. EPS Characterization

4.3.4.1. Colorimetric Analyses

Uronic acid content of the EPS was determined by the meta-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973, Filisetti-Cozzi and Carpita 1991). Protein content was determined by the Lowry protein assay (Lowry et al. 1951) with bovine serum albumin as the standard. The total neutral carbohydrate content was determined by the orcinol-sulfuric acid method modified by Rimington (1931).

4.3.4.2. Monosaccharide analyses

To a solution containing 250 µg total EPS, 50 µg erythritol was added as an internal standard. Samples for GC analysis were prepared in triplicate. The polymer was hydrolysed by the addition of methanolic HCl (3N, Supelco, PA, USA) and heating for 16 hr at 80°C (Kamerling et al. 1975, modified by Montreuil et al. 1986). The monosaccharides were converted to their trimethylsilyl derivatives by the addition of Bis-(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane / 99:1 (Supelco, PA, USA) and pyridine followed by incubation at room temperature for 2 hr. The samples were then dried under nitrogen and redissolved in dichloromethane prior to analysis.

4.3.4.3. Gas Chromatography

Analyses of the monosaccharides as trimethylsilyl derivatives were performed on a GC8000 (Fisons, France) gas chromatograph (GC) fitted with an automatic injector, a flame ionization detector (FID) and a CP-Sil-5CB glass capillary

column (0.32 mm x 60 m, Chrompac, Varian, France). Hydrogen was the carrier gas. The GC oven was temperature programmed as follows: 50°C for 1 min then an increase of 20°C/min until 120°C, followed by a gradient of 2°C/min until 240°C.

4.3.4.4. FT-IR Spectroscopy

Pellets for infrared analysis were obtained by grinding a mixture of 2 mg polysaccharide with 200 mg dry KBr, followed by pressing the mixture into a 16 mm diameter mold. The Fourier transform-infrared (FT-IR) spectra were recorded on a Bruker Vector 22 instrument with a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} region. Sulfate content was determined by FT-IR spectroscopy according to the method of Lijour et al. (1994).

4.3.4.5. NMR Spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AMX-500 (500 MHz for ^{1}H and 125MHz for ^{13}C) at 55°C. Samples were exchanged three times with D_2O with intermediate lyophilization and finally dissolved in 500 µl D_20 to a final concentration close to 30 mg. Chemicals shifts were reported in parts per million relative to sodium 2,2,3,3-d_4-(trimethylsilyl) propanoate for ^{1}H and CDCl3 for ^{13}C NMR spectra.

4.4. Results

4.4.1. Isolation and characterization of bacterial isolates

4.4.1.1. Cultivation of isolates

Bacterial strain CAM025 was isolated from filtered sea-ice particulates, while CAM036 was isolated from diatom-rich trawled material. Both strains showed enhanced growth on MA+Glu and, after 14 days of growth at 20°C on this medium, produced opaque, circular, convex or pulvinate, off-white colonies 1-2 mm in diameter with a mucoid texture and an entire margin. Both were Gram-negative curved rods (2-5 µm x 0.5 µm).

4.4.1.2. Whole cell fatty acid analyses

Table 4.1 contains a list of the whole cell fatty acids present in CAM025 and CAM 036 as the percentage of the total fatty acids. Major whole cell fatty acids in CAM025, in decreasing order of abundance, included 17:1ω8c, 16:1ω7c, 16:0, 17:1ω8t, 17:0, 15:0 and 15:1ω8c. For CAM036, the major whole cell fatty acids included 16:1ω7c, 17:1ω8c, 16:0, 16:1ω7t, βOH-12:0, 17:0, and 17:1ω8t. These results are generally consistent with those reported for *Pseudoalteromonas* strains from other studies (Bozal et al. 1997, Bowman 1998).

4.4.1.3. 16S rDNA sequences

Analysis of partial 16S rDNA sequences from CAM025 (1488 base pairs) and CAM036 (1485 base pairs) also indicated that both isolates belonged to the genus *Pseudoalteromonas,* with the closest species *P. haloplanktis* (sequence similarity CAM025: 99.3%, CAM036: 99.4%) and *P. nigrifaciens* (sequence similarity CAM025: 99.6%, CAM036: 99.8%). Figure 4.1 shows the phylogenetic relationship of *Pseudoalteromonas* species and two Antarctic isolates (CAM025 and CAM036) based on 16S rDNA sequences. The tree was created using maximum-likelihood distanced clustered by the neighbor-joining method (Bar: 0.1 changes per mean nucleotide position). Numbers in parentheses are GenBank nucleotide accession numbers. Outgroup for the analysis was *Escherichia coli* (J01695). Precise species identification of CAM025 and CAM036 will require further investigation by DNA-DNA hybridization.

4.4.2. Characterization of exopolysaccharides

4.4.2.1. Colorimetric analyses

The gross chemical composition of bacterial EPS is presented in Table 4.2. Protein content for EPS from CAM025 and CAM036 was low (2-3%). Neutral sugars dominated in both EPS and accounted for 74% and 50% of EPS from CAM025 and CAM036, respectively. Both EPS contained significant amounts of uronic acids (CAM025 – 22%; CAM036 – 25%) according to colorimetric analyses.

Table 4.1. Whole cell fatty acids of two Antarctic marine bacterial isolates^ reported as a percentage of total area

Fatty Acid	CAM025	CAM036
i14:0 + β-OH i12:0	1.5	2.3
14:0	tr	tr
β-OH 12:0	3.5	6.5
i15:1	1.9	2.1
15:1ω8c	4.0	3.3
15:1ω8t	1.5	1.8
15:0	4.7	3.3
β-OH 13:0	2.2	3.3
i16:0	tr	tr
16:1ω9c	tr	tr
16:1ω7c	21.4	26.2
16:1ω7t	7.5	6.7
16:0	7.5	7.3
17:1ω8c	29.3	24.2
17:1ω8t	6.8	4.1
17:0	4.8	5.6
18:1ω7c	3.5	3.3
Total	100	100

^Grown at 12°C for 4 weeks on Marine Agar
tr: %≤0.5% total area

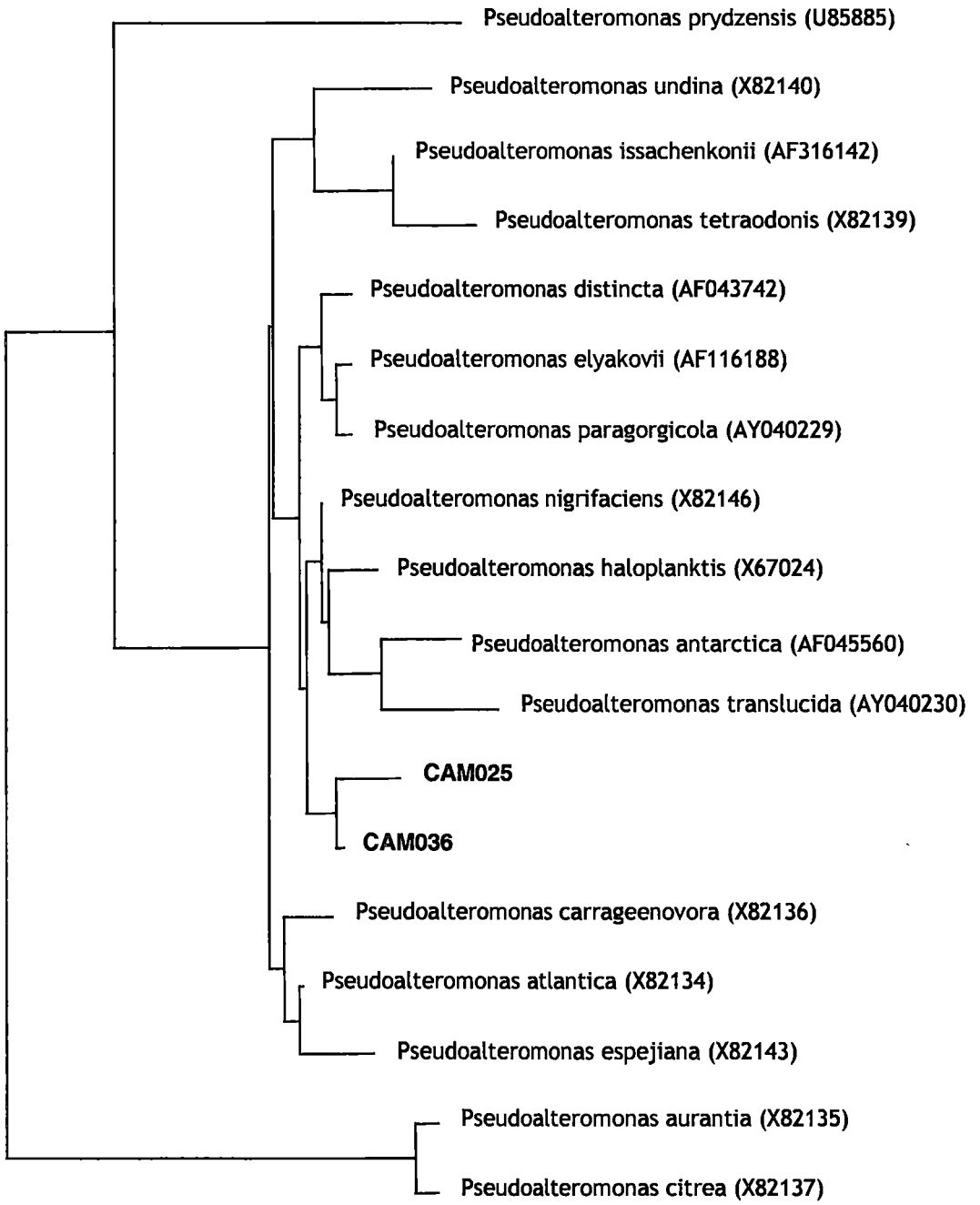

Figure 4.1. Phylogenetic relationship of *Pseudoalteromonas* species and two Antarctic isolates (CAM025 and CAM036) based on 16S rDNA sequences. The tree was created using maximum-likelihood distanced clustered by the neighbor-joining method. Bar 0.1 changes per mean nucleotide position. Numbers in parentheses are GenBank nucleotide accession numbers. Outgroup for the analysis was *Escherichia coli* (J01695).

4.4.2.2. FT-IR analyses

FT-IR spectra of the CAM025 and CAM036 EPS (Figure 4.2) displayed a broad O-H stretching band above 3000 cm^{-1} and intense absorptions between 1650 and 1050 cm^{-1} characteristic of polysaccharides. An absorbance at approximately 1730 cm^{-1} indicated the presence of carboxyl groups (Lijour et al. 1994). In addition, a small absorption at 1550 cm^{-1}, indicative of either amino sugars or proteins was

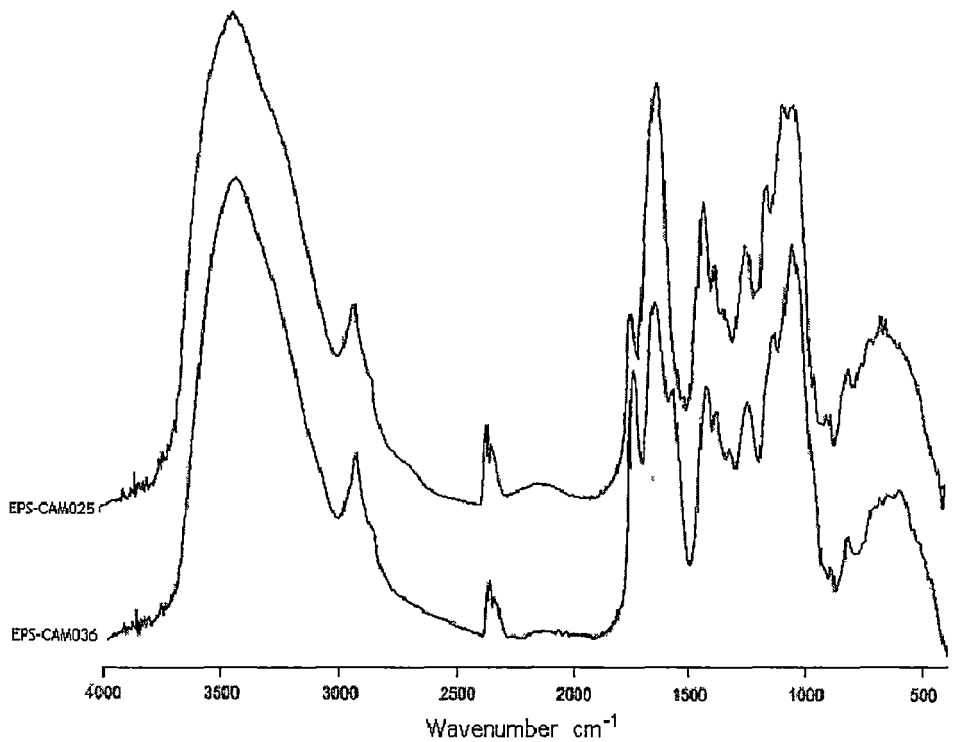

Figure 4.2. FT-IR spectra of EPS from CAM025 and CAM036, two Antarctic marine bacteria grown in laboratory culture. Absorbances at 3000-3600cm^{-1} are indicative of OH stretch; 1730 cm^{-1}, carboxyl groups; 1650 cm^{-1} and 1050 cm^{-1}, polysaccharides; 1550 cm^{-1}, amino sugars and/or proteins; 1230-1250 cm^{-1}, sulfate.

present in CAM036 EPS, but extremely small in CAM025 EPS. GC data confirmed the presence of amino sugars in the CAM036 EPS (Table 4.3) and minor amounts in the CAM025 EPS. Sulfate content in each EPS, determined by the presence of a doublet at 1230-1250 cm-1, was estimated to be 5% for each of the EPS samples (Table 4.2).

Table 4.2. Chemical analysis of exopolysaccharides from two Antarctic bacterial marine isolates (g/100 g total EPS)

Isolate	Uronic Acids (%)	Neutral Sugars (%)	Proteins (%)	Sulfates (%)
CAM025	22	74	2	5
CAM036	25	50	3	5

Table 4.3. Molar ratios of EPS monosaccharides* determined by GC-FID analysis after acid methanolysis and trimethylsilyl derivatization. (Values have been normalized to Ara =1.0).

	Isolate	
Monosaccharides	CAM025	CAM036
Ara	1.0	1.0
Rib	0.3	0.0
Rha	1.1	0.0
Fuc	0.4	0.0
Gal A	6.4	7.2
Man	0.3	5.8
Gal	1.3	0.4
Glc	12.8	6.1
Gal N Ac	0.1	2.6

*Ara indicates arabinose; Rib, ribose; Rha, rhamnose; Fuc, fucose
Xyl, xylose; Gal A, galacuronic acid; Glc A, glucuronic acid;
Man, mannose; Gal, galactose; Glc, glucose;
Gal N Ac, N-acetyl-galactosamine

4.4.2.3. Monosaccharides analyses

Molar ratios of monosaccharides are listed in Table 4.3. The EPS secreted by the bacterial strain CAM025 was composed of five major monosaccharides with glucose predominating over other neutral sugars (arabinose, galactose) and traces of ribose, fucose and mannose were also present. The presence of galacturonic acid was also substantial, accounting for approximately one-quarter of the monosaccharides in the EPS. The EPS produced by bacterial isolate CAM036 was composed of five monosaccharides with galacturonic acid predominating over the other sugars accounting for one third of the monosaccharides present. Mannose and glucose were the most abundant neutral sugars along with arabinose while galactose was present as traces. N-acetyl galactosamine was a relatively abundant aminyl-sugar in this EPS

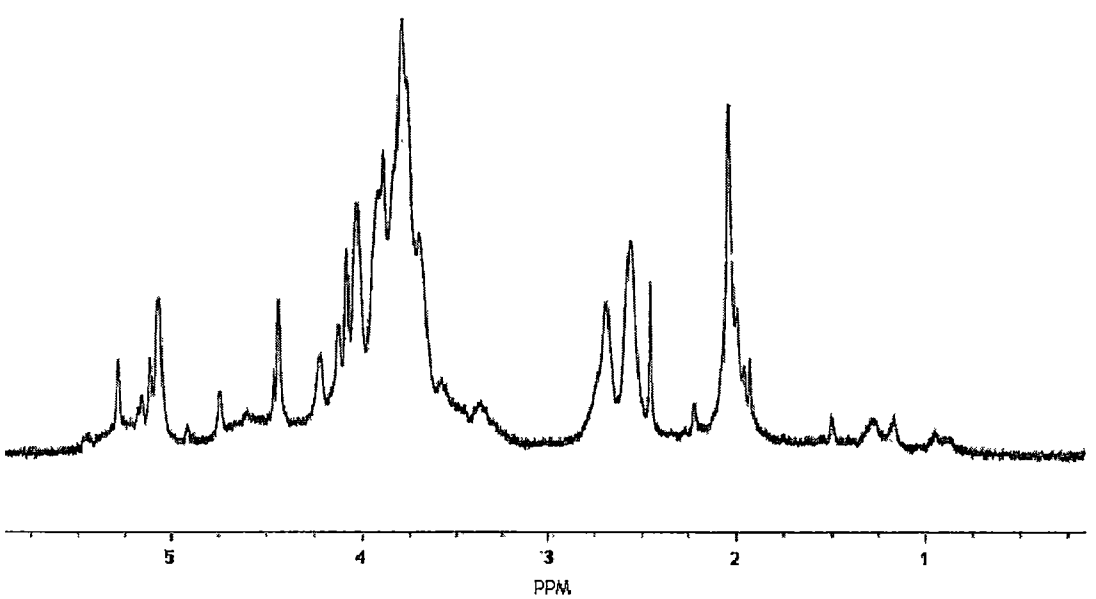

Figure 4.3. ^{1}H NMR spectra of EPS produced in laboratory culture by Antarctic marine bacterial isolate, CAM036. Signal at δ 2.02 ppm indicates acetyl group, the signals at δ 2.55 ppm and δ 2.68 ppm, indicate a succinyl group.

4.4.2.4. NMR Spectra

From the ^{1}H and ^{13}C NMR spectra of the native EPS, it is possible to extrapolate some information. The ^{1}H spectrum showed essentially five and seven anomeric signals for the EPS produced by isolates CAM025 and CAM036 respectively. The presence in both EPS of acetyl groups linked to different sugars was confirmed by a signal at δ 2.02 ppm. Interestingly, the signals at δ 2.55 ppm and δ 2.68 ppm, indicative of a succinyl group, were present only for the EPS produced by isolate CAM036. The ^{1}H NMR spectra for the EPS from CAM036 are presented in Figure 4.3.

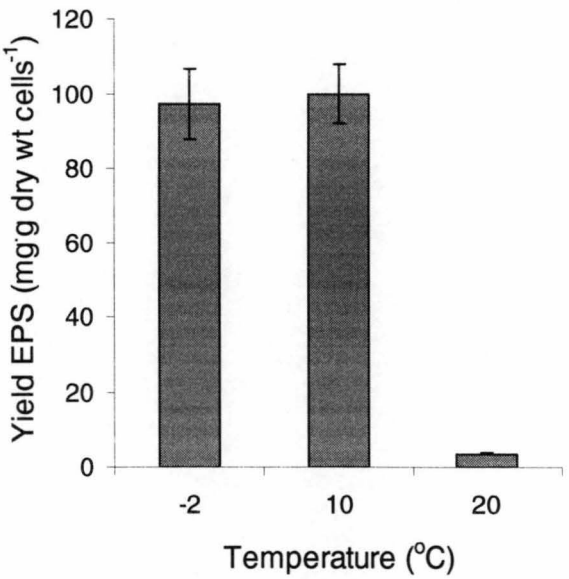

Figure 4.4.Yield of EPS (mg EPS per g dry weight of cells) from batch cultures CAM025 incubated at –2°C, 10°C and 20°C.

4.4.3. EPS yield determinations for CAM025

The average change in pH over the period of incubation of two CAM025 cultures at each of the three temperatures was calculated. After two weeks at –2°C and 10°C, the average decrease of 0.63 and 0.93 pH units respectively, was observed. After one week at 20°C, the average decrease in pH for these two cultures was 1.69 units. The final yields of EPS for CAM025 incubated at –2°C, 10°C and 20°C (Figure 4.4) were calculated by dividing the final weight of the freeze-dried polymer for each culture by the weight of the corresponding freeze-dried cell pellets. Results show that the yield of EPS for CAM025 grown at -2°C and at 10°C was approximately 100 mg EPS per gram dry weight of cells. The yield of EPS for the same strain grown at 20°C was approximately thirty fold lower.

4.5. Discussion

The bacterial strain CAM025 was obtained from particles from melted Antarctic sea ice retained on a filter with a pore size of 0.8 μm. CAM036 was isolated from particles captured by a plankton net (20 μm) towed through the Southern Ocean. Both psychrotolerant strains (growth at 4°C and 25°C) displayed an enhanced mucoid morphology on marine agar supplemented with glucose. Results from 16S rDNA sequencing and whole cell fatty acid analyses indicate that these two isolates were closely related and belonged to the genus *Pseudoalteromonas.*

Previous studies have shown that many *Pseudoalteromonas* strains are the psychrotrophic bacteria with a temperature growth range from 4°C to 30°C (Bozal et al. 1997, Bowman 1998), and show optimal growth at 22°C to 25°C (Bowman 1998). Members of this genus are the bacteria most frequently isolated from sea ice and underlying sea water (Bowman et al. 1997c, Bowman 1998;Delille 1992). In liquid culture amended with glucose, CAM025 and CAM036 produced EPS, which when chemically analyzed by colorimetric techniques were shown to have similarly low amounts of protein and abundant neutral sugars and uronic acids. FT-IR and NMR analyses confirmed the presence of acetyl groups and low amounts of ester sulfate groups in both polysaccharides. Similar biochemical compositions were observed in previous studies of EPS from *Alteromonas* species

isolated from hydrothermal vent communities (Rougeaux et al. 1996, Cambon-Bonavita et al. 2002, Raguénès et al. 2003).

Arctic studies (Krembs and Engel 2001, Krembs et al. 2002) have shown that large quantities of microbially produced EPS occur in sea ice and at the ice-water interface. This material was positively correlated to bacterial abundances although diatoms were thought to dominate the EPS production in this system. These authors suggest high concentrations of EPS in the brine channels may provide buffering against harsh winter conditions and high salinity as well as cryoprotect the microbes living there against ice crystal formation. In our study, the EPS yield data suggest that there is a decreased production of EPS at higher temperature (20°C) for the Antarctic sea ice strain tested. This finding supports the proposed hypothesis that EPS production by psychrotolerant bacteria may play an important role in the sea ice microbial community.

The Arctic sea ice studies (Krembs and Engel 2001, Krembs et al. 2002) also demonstrated that the neutrally buoyant polymeric material was carried large distances by prevailing under-ice currents and ice drifts. Studies in more temperate waters show marine bacterial EPS production plays a major role in the aggregate formation process (Biddanda 1986, Decho 1990). When released into the water column, a combination of biological, chemical and physical forces causes this colloidal material to form aggregates (Alldredge and Jackson 1995, Passow 2000, Kiorboe 2001), which become centers of high microbiological heterotrophic activity (Kiorboe 2001).

Preliminary characterizations show that the structure of the EPS from CAM025 and CAM036 includes sulfate as well as high levels of uronic acids as galacturonic acid, along with acetyl groups. In addition, the EPS from CAM036 was shown by NMR data to include a succinyl group. These features convey an overall polyanionic or 'sticky' quality to the EPS in the marine environment, since at the pH of seawater (pH 8.0) many of the acidic groups present on these polymers are ionized (Decho 1990). This 'stickiness' is important in terms of the

affinity of these EPS for binding to other cations such as dissolved metals (Brown and Lester 1982).

The EPS were subjected to further analytical characterization and based on relative molar ratios of monosaccharides (normalized to arabinose), there were several similarities between these two EPS including the presence of the sole acidic sugar, galacturonic acid in significant proportion. Glucose was a major neutral sugar present in both polysaccharides, albeit to varying degrees. There were also several differences between the EPS from the two Antarctic strains. Rhamnose and fucose were present in the CAM025 EPS and absent in CAM036 EPS. Mannose accounted for a large proportion in the CAM036 EPS while being present at low levels in the EPS produced by CAM025, based on molar ratios. Another significant difference is the presence of aminyl sugars in only one polysaccharide (CAM036) as determined by both the GC and NMR analyses.

In this study we have demonstrated that two Antarctic marine bacterial isolates; one from Southern Ocean particulate material and the other from melted sea ice, belong to the genus *Pseudoalteromonas* and produce different EPS in laboratory culture. This feature is also found in bacteria from deep-sea hydrothermal vents (Rougeaux et al. 1996, Raguénès et al. 1997b) and other aquatic microbial communities (Nicolaus et al. 1999). The EPS produced by Antarctic bacterial isolates examined in this study appear to be polyanionic and, therefore, 'sticky' with respect to cations such as trace metals. The availability of iron as a trace metal is of critical importance in the Southern Ocean where it is know to limit primary production (Scharek et al. 1997). Since 99% of dissolved iron in the ocean is bound to organic ligands (Rue and Bruland 1995), implications for the role of these bacterial polysaccharides in the Antarctic marine environment require further investigation.

Biotechnological uses for microbially produced EPS include environmental, clinical, nutritional and cosmetic applications, to name a few (Guezennec 2000, Gutnick and Bach 2000, Sutherland 2001). Increased knowledge of the role of

Antarctic bacterial EPS will also provide insight into possible commercial uses for these novel polymers.

4.6. ACKNOWLEDGEMENTS

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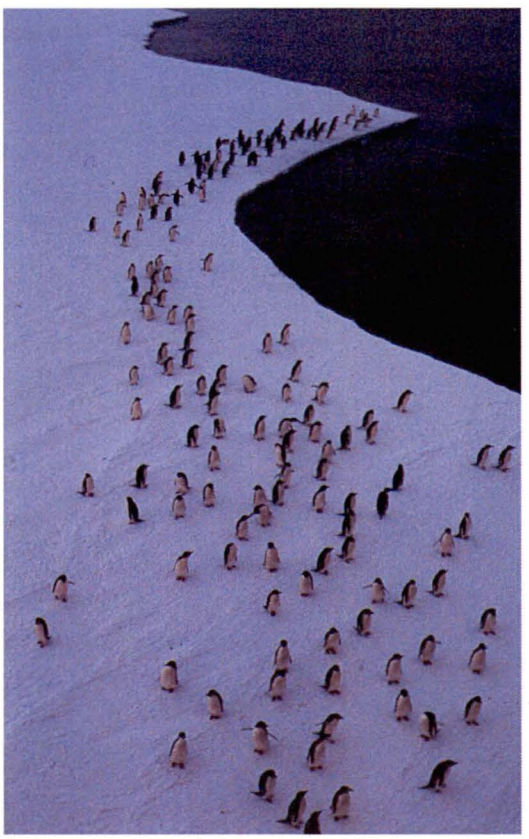
Chapter 5. The effect of incubation temperature on the growth and production of exopolysaccharides by an Antarctic sea ice bacterium grown in batch culture



Chapter 5. The effects of incubation temperature on the growth and production of exopolysaccharides by an Antarctic sea ice bacterium grown in batch culture

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The research within the original manuscript and this resulting Chapter was conducted by myself. Co-authors John Bowman and Jean Guezennec fulfilled supervisory roles.

5.1. ABSTRACT

The sea ice microbial community plays a key role in the productivity of the Southern Ocean. Exopolysaccharide (EPS) is a major component of the sugar-containing exopolymer secreted by many marine bacteria to enhance survival and is abundant in sea ice brine channels, but little is known about its function there. This study investigated the effects of temperature on EPS production in batch culture by CAM025, a marine bacterium isolated from sea ice sampled from the Southern Ocean. Previous studies have shown that CAM025 is a member of the genus *Pseudoalteromonas*, and therefore belongs to a group found to be abundant in sea ice by culture dependent and independent techniques. Batch cultures were grown at -2°C, 10°C and 20°C and cell number, optical density, pH, glucose concentration and viscosity were monitored. The yield of exopolymer at -2°C and 10°C was 30 times higher than at 20°C, which is the optimum growth temperature

for many psychrotolerant strains. Exopolymer may have a cryoprotective role in brine channels of sea ice, where extremes of high salinity and low temperature impose pressures on microbial growth and survival. EPS produced at -2°C and 10°C had a higher uronic acid content than that produced at 20°C. The availability of iron as a trace metal is of critical importance in the Southern Ocean where it is known to limit primary production. EPS from strain CAM025 is polyanionic and may bind dissolved cations such as trace metals therefore the role of bacterial EPS in the Antarctic marine environment may have important ecological implications.

5.2. INTRODUCTION

Sea ice is a major component of polar regions, covers millions of km^2 even at the end of the polar summer (Horner et al. 1992), and provides a home to unique communities dominated by microorganisms (Staley and Gosink 1999). Sea ice begins to form as the temperature of seawater, with a salinity of 3.5% drops to –1.8°C. As ice develops, a salting-out process occurs in which the marine salts excluded from the ice are concentrated in brine pockets. Brine channels develop as the ice rises above sea level. The dense brine drains through the layer of columnar congelation ice by gravity and flows to the underlying seawater (Brierley and Thomas 2002). The sea ice produces highly variable microenvironments in terms of temperature, salinity, nutrient concentration and light intensities within the columns, which may be as thick as 2 m. Salinity in sea ice brine can range from near that of freshwater to >15% at the ice-sea water interface (Staley and Gosink 1999). Temperatures can range from 0 °C to –35°C. These factors contribute to sea-ice being one of the coldest habitats on earth for marine life (Junge et al. 2002).

In spite of these extremes, up to 20-30% of primary production in sea ice cycles through heterotrophic bacteria (Kottmeier et al. 1987, Palmisano and Garrison 1993). Complex microbial communities, dominated by diatoms in close association with bacteria (Palmisano and Garrison 1993), are concentrated in the lower 10-20 cm of sea ice where nutrients are available from the sea water and light is available from the surface (Staley and Gosink 1999). Abundant bacterial

populations have been found in thick annual pack ice with psychrophilic bacteria being particularly common in samples of pore waters and ice colored brown by the presence of algal pigments (Delille 1992). During ice formation microalgal cells are scavenged by sea ice crystals floating up to the sea surface (Gleitz and Thomas 1993), and bacteria attached to algal cells may be incorporated into new ice in conjunction with some algal species (Grossmann and Dieckmann 1994). Bacterially-produced exopolymer may provide a means by which bacteria can adhere to the microalgal cells (Sullivan and Palmisano 1984). Studies of both the Arctic (Krembs and Engel 2001) and Antarctic (Sullivan and Palmisano 1984) sea ice communities suggest that exopolymer production by both phytoplankton and bacteria make a significant contribution to organic carbon in the sea-ice and ice-water interfaces.

Little is known about EPS produced by Antarctic marine bacteria or about its role in this extreme environment. Sea ice bacteria maintained in laboratory culture can secrete copious amounts of mucus (Helmke and Weyland 1995, Mancuso Nichols et al. 2005c). The sea ice isolate CAM025, examined in previous studies belongs to the genus *Pseudoalteromonas* and class "*Gammaproteobacteria*" (Mancuso Nichols et al. 2004, Mancuso Nichols et al. 2005c). Studies of polar sea ice communities using cultivation dependent and independent techniques have shown that the "*Gammaproteobacteria*" are among the dominant taxonomic groupings (Bowman et al. 1997c, Staley and Gosink 1999, Brown and Bowman 2001, Brinkmeyer et al. 2003). CAM025 exhibits growth in the temperature range -2°C to 25°C, on media containing 1% to 12% (w/v) sea salts and exhibits an enhanced mucoid morphology on marine media with added glucose (Mancuso Nichols et al. 2004).

When grown in batch culture at temperatures near the predicted optimum for this strain, CAM025 produced exopolymer and chemical analyses showed the purified EPS was composed primarily of neutral sugars (glucose, arabinose, fucose and galactose), uronic acids (glucuronic acid) and sulfates (Mancuso Nichols et al. 2004). Sulfates carry a net negative charge at seawater pH (Leppard et al. 1996); uronic acids also contain an acidic carboxyl group that is ionisable in these

conditions. The presence of these two groups (uronic acids and sulfates) is known to result in an EPS with a polyanionic quality (Decho 1990). Due to these chemical characteristics, microbial exopolymer like those produced by CAM025 have been shown to accumulate cations such as metals (Bitton and Friehofer 1978, Brown and Lester 1982). The ecological role of EPS is, therefore directly related to its chemistry. The frequency and type of functional groups present in the EPS impact on the tertiary structure and over-all physico-chemical characteristic of the polymer in the surrounding aqueous environment (Decho 1990).

EPS of strain CAM025 had a molecular weight of 5700 Da, which is high relative to EPS produced by many other marine bacteria (100 to 300 kDa, Decho 1990) but similar to molecular weights of EPS produced by bacteria from deep-sea hydrothermal vents (Guezennec 2002). The physical, rheological and chemical properties of the structure and properties of EPS are influenced by the length of the polymer chain, that is, the molecular weight (Christensen 1999). As the length of the polymer increases, there is a greater opportunity for complex entanglement of polymer chains and intramolecular associations, and these contribute to the tertiary structure and physical behavior of the polymer (Sutherland 1994). A fungal strain, *Phoma herbarum,* isolated from Antarctic soil produced a homosaccharide of glucose with a molecular weight of 7400 kDa (Selbmann et al. 2002).The authors suggested the fungal EPS could provide a cryoprotective role in the harsh Antarctic environment where the availability of liquid water and temperatures are extremely low.

Exopolymer produced by CAM025 in laboratory cultures incubated at 20°C, has provided some insight into the possible ecological role for these biopolymers. Although 20°C is near the predicted optimum for growth of this psychrotolerant strain, it is much higher than that of the natural environment from which this strain was isolated. This study was undertaken to investigate the effects of a range of temperatures on the production of exopolymer by sea ice isolate *Pseudoalteromonas* strain CAM025.

5.3. Materials and Methods

5.3.1. Batch cultures at different temperatures

The CAM025 was grown in duplicate broth cultures at -2°C, 10°C and 20°C. Culture broth was composed of 1 g yeast extract, Oxoid L21; 5 g bacteriological peptone, Oxoid L37; 32 g artificial sea salts, Sigma S9883; 4.76 g HEPES, Sigma H7637; and 1000 ml distilled water. The pH of the broth was adjusted to 8 prior to autoclaving. A glucose solution was prepared and autoclaved separately before being combined with the above media for a final concentration of 3% glucose (w/v). One litre baffled flasks containing 220 ml of the above media were inoculated with 20 ml of the exponentially growing batch cultures of the bacterial isolate. Approximately 5 ml of the broth culture was removed aseptically from each of the flasks to sterile McCartney bottles for pH, optical density (600 nm), viable cell counts, viscosity and glucose measurements (described below) at inoculation and then once daily through the experiment. Flasks were incubated in oscillating water baths (Ratek Pty Ltd, Australia) fitted with refrigeration units that cooled and circulated antifreeze liquid at the desired temperature. All incubations took place in a room maintained at 20°C. Batch cultures at -2°C, 10°C were harvested after 2 weeks incubation. The cultures at 20°C were harvested after 1 week.

5.3.2. Enumeration

Optical density of culture material was measure at 600 nm at 24 hr intervals using a Smart Spec 3000 spectrophotometer (Bio-Rad, Regent Park, NSW, Australia). Aliquots (1 ml) of culture media were removed from flasks at 48 hr intervals, serially diluted in sterile artificial sea salts solution (3.2% w/v in distilled water) and aliquots (150 µl) were spread onto replicate agar plates (Marine agar with 3% (w/v) added glucose; MA+Glu) with a Spiral Biotech automated plater (Bethesda, USA). Cultures were incubated at 12°C for 4 days before colonies were enumerated for viable cell count determination. The pH of culture material at 20°C was measured at 24 hr intervals with an Orion pH electrode (Boston, USA)

5.3.3. Chemical and physicochemical measurements

The viscosity of a one ml aliquot of the culture broth, warmed to room temperature (20°C), was measured daily with a Brookfield LVT microviscometer fitted with a cone and plate assembly. (Middleboro, MA, USA). One ml aliquots of culture broth, removed every 24 hr, were centrifuged at 15,000 g for 10 min (Eppendorf, Hamburg, Germany). The supernatant was removed and frozen. Glucose was quantified using a Boehringer Mannheim kit (Darmstadt, Germany).

5.3.4. Isolation and purification and characterization of CAM025 EPS

Culture broth was centrifuged at 30,000 g for 2 hr at 4°C (Beckman Coulter, Pasadena, USA). The cell pellets were freeze-dried and weighed. EPS was purified, uronic acid, protein and total neutral carbohydrate content of the exopolymer was determined and analysis of EPS monosaccharides was performed according to procedures described in detail (Mancuso Nichols et al. 2004).

5.3.5. Statistical treatment of data.

Univariate analysis of variance was performed on data gathered from measurements of cell yield, exopolymer or EPS yield, glucose consumption (initial glucose concentration minus final glucose concentration; $[Glu]_{init} - [Glu]_{final}$), crude chemical analysis and monosaccharide analyses of EPS. The statistical analysis was carried out using General Linear Model package of SPSS (SPSS 2004, all rights reserved).

5.4. RESULTS

5.4.1. Growth of CAM025 in batch cultures

Viable cell counts showed that the highest rate of exponential growth occurred in cultures incubated at 10 and 20°C in the first 24 hr (Figure 5.1). Cultures at -2°C reached a maximum viable cell count by day 5 of incubation. The final optical density reading (600 nm) for each culture was used as an indication of the cell

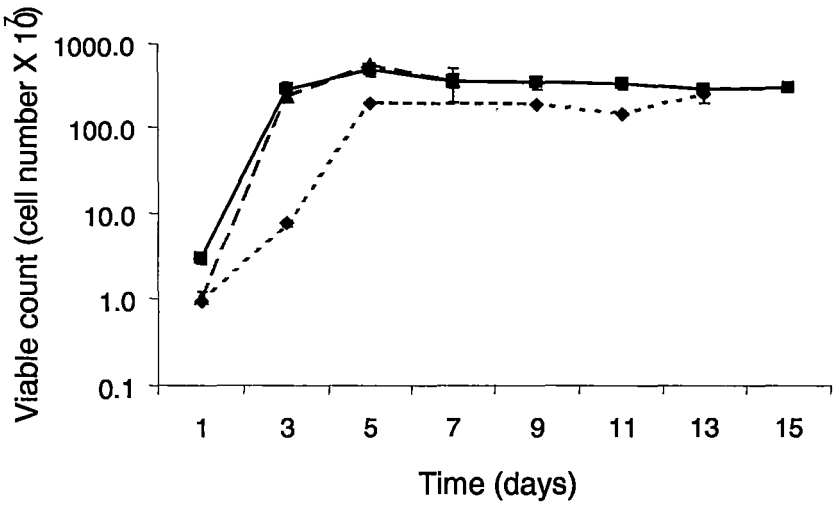

Figure 5.1. Viable cell count for batch cultures of sea ice isolate CAM025, incubated at -2°C (diamond) 10°C (square) and 20°C (triangle).

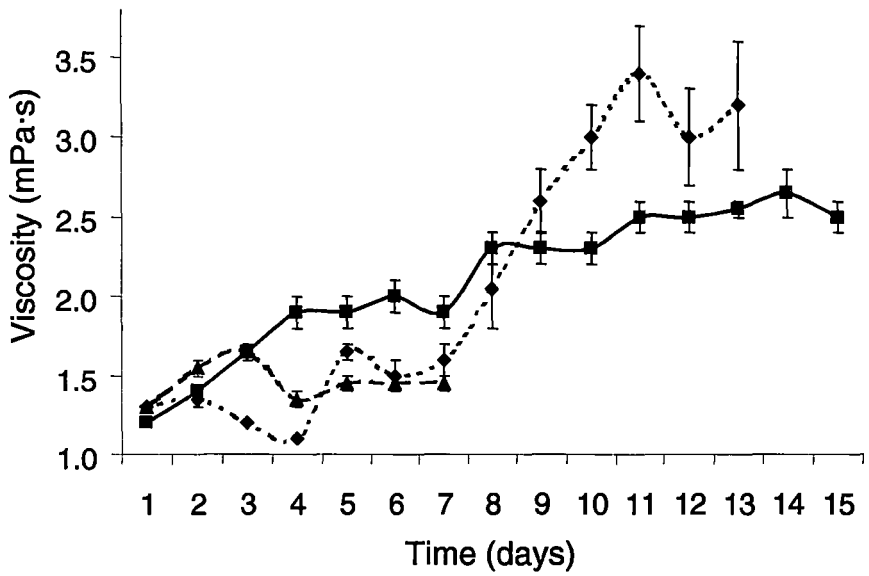

Figure 5.2. Viscosity for batch cultures of sea ice isolate CAM025, incubated at -2°C (diamond) 10°C (square) and 20°C (triangle).

yield. The cell yield was significantly different between cultures incubated at -2°C and 10°C (Table 5.1).

Viscosity of culture media from 20°C incubations reached a maximum of 1.7 mPa·s after 72 hr (Figure 5.2). A decrease on day four was followed by no change on the subsequent three days. Viscosity of cultures at 10°C increased gradually to a maximum of 2.7 mPa·s on day 14 and then decreased slightly. Cultures incubated at -2°C showed the greatest increase in viscosity, reaching a maximum of 3.4 mPa·s after 11 days.

The pH of cultures at 10°C decreased 0.7 pH units within the first 24 hr of incubation then decreased gradually until day 15 (Figure 5.3). The pH of cultures incubated at -2°C decreased gradually over the first six days and then gradually increased until day 13. The pH of 20°C cultures decreased 1.5 pH units by day three and after a slight increase on day four and five, decreased over the subsequent two days. This decrease in pH was occurring without increase in viscosity. The -2°C and 10°C cultures, in comparison, showed increases in viscosity and smaller decreases in pH (Figure 5.3).

The concentration of glucose in culture media was measured throughout the experiment (Figure 5.4). The difference in glucose concentration in the batch cultures between the start and end of the incubation was calculated to represent the amount of glucose consumed at each temperature. Maximum glucose consumption occurred in cultures incubated at 10°C, where the final glucose concentration was 80% lower than at the start of the experiment. In contrast, a glucose consumption of 15% occurred in cultures incubated at -2°C. The difference in values representing glucose consumption at these two incubation temperatures was significant (Table 5.1).

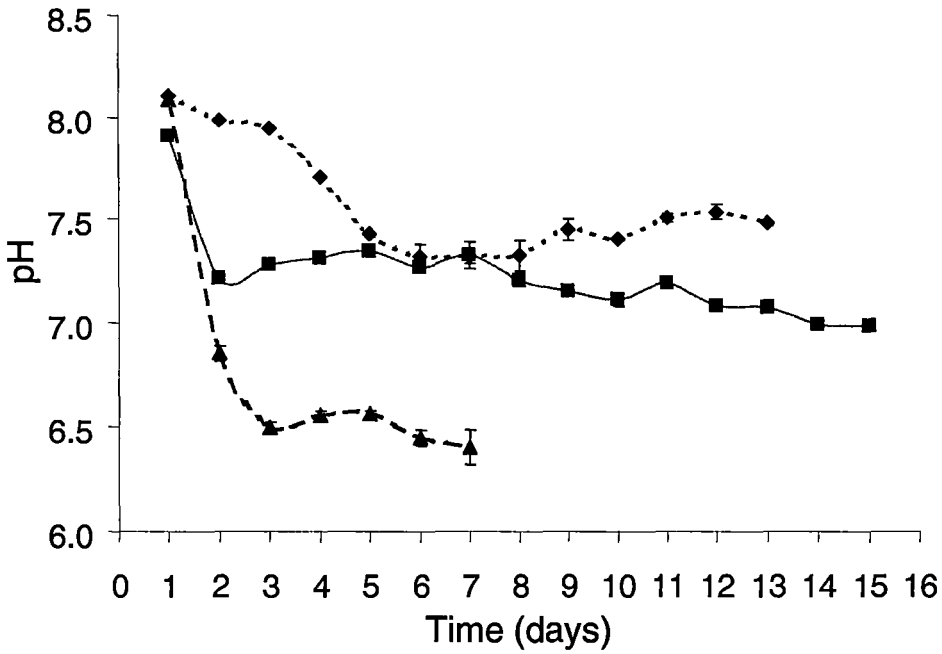

Figure 5.3. pH for batch cultures of sea ice isolate CAM025, incubated at -2°C (diamond) 10°C (square) and 20°C (triangle).

5.4.2. Production of exopolymer

Exopolymer harvested from each culture at the end of the experiment was purified by centrifugation, filtration, ethanol precipitation and dialysis as described previously (Mancuso Nichols et al. 2004), dried and weighed. Cell material was harvested, freeze-dried and weighed. Yield of EPS at each temperature is expressed as mg EPS per g dry weight of cell material. The yield of EPS at -2°C and 10°C was approximately 30 fold higher than at 20°C (Table 5.1).

5.4.3. Characterization of EPS

The crude chemical composition of EPS produced by replicate cultures incubated at three temperatures is shown as a percentage of the total (Figure 5.5).

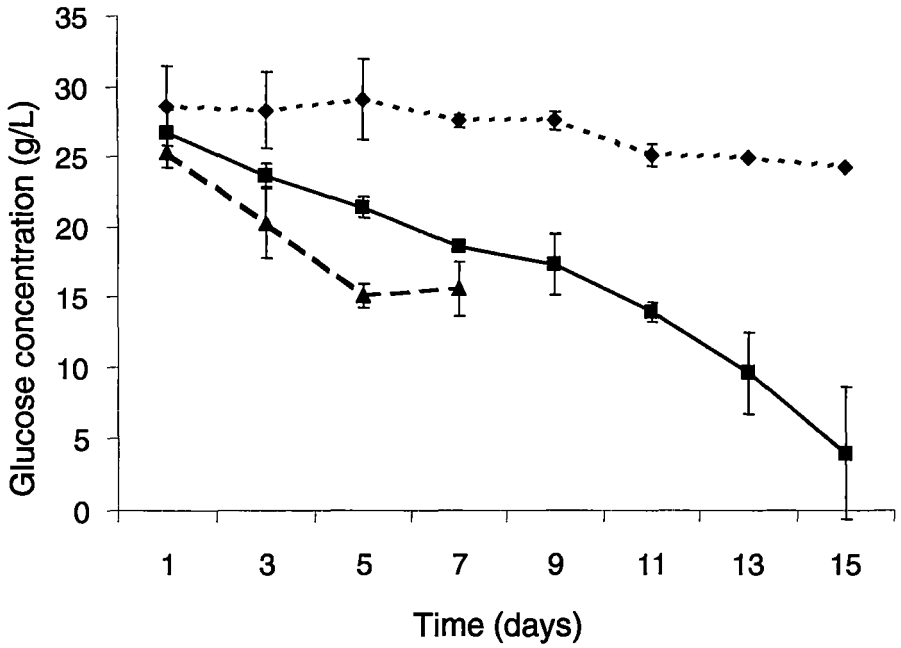

Figure 5.4. Glucose concentration for batch cultures of sea ice isolate CAM025, incubated at-2°C (diamond) 10°C (square) and 20°C (triangle).

Differences in neutral sugar and protein content in the EPS produced by cultures at the three incubation temperatures were not significant. In contrast, uronic acid content in EPS expressed as percentage of total EPS produced at -2°C and 10°C was significantly different ($p < 0.05$) from and higher than at 20°C. Analysis of individual monosaccharides in EPS harvested from duplicate cultures incubated at -2°C, 10°C and 20°C did not reveal any significant differences in the percentages of ribose, fucose and arabinose. Percentages of mannose, glucose and galactose were higher in cultures incubated at -2°C than at 10°C or at 20°C, whereas percentages of galactose and rhamnose were lower at -2°C than at 10°C or at 20°C. Percentages of galacturonic acid were lower in cultures incubated at 20°C than in those incubated at 10°C or at –2°C (Table 5.2).

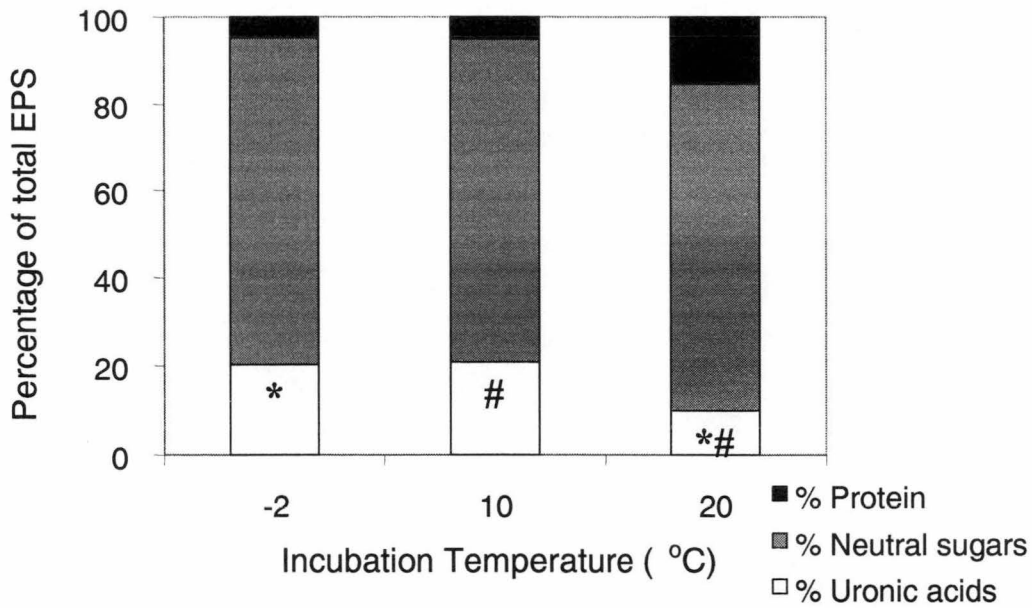

Figure 5.5. Crude chemical analysis of EPS produced by sea ice isolate CAM025 produced in batch cultures incubated at -2°C, 10°C and 20°C, reported as percentage of total EPS. Pairs of symbols * and # denote significant ($p < 0.05$) differences in percentages.

Table 5.1. Growth parameters of sea ice bacterium *Pseudoalteromonas* strain CAM025 grown in duplicate batch cultures

		Incubation temperature					
Parameter	Units	-2°C		10°C		20°C	
		Ave	SE	Ave	SE	Ave	SE
Cell yield	Final Absorbance (600 nm)	4.68[a]	0.3	10.13[a]	0.4	7.7	1.0
EPS yield	mg/g dry weight cells	97.2[b]	9.3	99.9[c]	8.0	3.6[b,c]	0.2
Glucose consumption $[Glu]_{initial} - [Glu]_{final}$	g/L	4.10[d]	2.5	22.8[d]	2.9	9.7	0.8

[a] Significant difference ($p \leq 0.071$), [b] ($p \leq 0.024$), [c] ($p \leq 0.024$), [d] ($p \leq 0.076$)

5.5. Discussion

Previous studies reported that the sea ice bacterium *Pseudoaltermonas* strain CAM025 exhibited growth in the temperature range -2 to 25°C but no growth occurred on solid media at 37°C (Mancuso Nichols et al. 2004). Other closely related *Pseudoalteromonas* species were shown to be psychrotolerant, that is, able to grow at 4°C and with a growth temperature optimum of approximately 22-25°C

(Bowman 1998). Psychrotolerant bacteria appear to be common in both sea ice and in underlying water (Brown and Bowman 2001, Delille 1996, Helmke and Weyland 1995) with *Pseudoalteromonas* strains being the most frequently isolated within this group (Bowman et al. 1997c).

Table 5.2. Monosaccharide composition of EPS from duplicate cultures incubated at three temperatures. Data expressed as percentage of total.

	Incubation temperature		
Monosaccharides	-2°C	10°C	20°C
Arabinose	3.1 (1.0)	3.7 (1.9)	11.3 (2.5)
Ribose	1.2 (0.9)	1.6 (0.2)	0.0 (0.0)
Rhamnose	4.8 (0.5)[b,c]	18.8 (3.1)[b]	25.9 (5.8)[c]
Fucose	1.6 (0.4)	7.7 (0.4)	6.9 (2.9)
Galacturonic Acid	15.8 (0.4)[e,f]	8.1 (1.0)[e,g]	0.2 (0.2)[f,g]
Mannose	16.5 (0.7)[h,j]	9.6 (1.5)[h]	8.9 (0.8)[j]
Galactose	5.7 (0.3)[k,l]	19.7 (3.2)[k]	23.6 (2.0)l
Glucose	51.3 (1.8)[m,n]	30.9 (5.4)[m]	23.3 (9.2)[n]

[a] Standard errors of the means are given in parentheses.

[b-n] Pairs of letters denote significant differences ($p < 0.05$) betweens percentages of EPS monosaccharides from duplicate cultures.

5.5.1. Cell yield, EPS yield and glucose consumption

In 10°C cultures, exponential growth phase occurred in the first 24 hours and was followed by a stationary phase in which viscosity increased. Cultures grown at 10°C also showed maximum optical density readings, highest cell yield, yield of EPS and glucose utilization compared to cultures at -2°C and 20°C (Table 5.1). A high yield of EPS accompanied by high cell yield appears to indicate balanced growth was occurring at this temperature compared to -2°C and 20°C. Maximum viscosity was reached during stationary phase in all cultures, with cultures growing at -2°C and 10°C showing the highest viscosity.

In batch culture studies of deep-sea hydrothermal vent isolate, HYD 1545, EPS production began in late exponential phase and continued during stationary phase (Vincent et al. 1994). Deep-sea hydrothermal vent strain *Alteromonas* sp. strain 1644 produced an EPS at the beginning of stationary phase and this suggested that synthesis was induced by restricted growth conditions (Samain et al. 1997). In other experiments with mesophilic lactic acid bacterium *Lactobacillus delbrueckii*, EPS was produced during stationary phase (Petry et al. 2000). The authors also observed that maximum bacterial growth produced optimal EPS yield and most glucose utilization occurred during stationary phase. In the current study, cultures of CAM025 incubated at 20°C, pH decreased by 1.7 units while viscosity remained low during early stationary phase. EPS production at 20°C seems to be restricted compared to production of EPS in cultures incubated at -2°C and 10°C.

In growth experiments with *Lactobacillus sakei* strain 0-1, low temperature combined with glucose as a carbohydrate source enhanced EPS production (Degeest et al. 2001). In the observed growth temperature range of 15-42°C, 15 and 25°C were the best for bacterial growth and EPS production for this mesophile. Specific production of EPS decreased with increasing temperature. Depletion of glucose indicated the C/N ratio of the media was optimal (Degeest et al. 2001). In the current study, glucose was depleted by 80% in cultures incubated at 10°C, and EPS and cell yield were the highest at this temperature. Cultures at -2°C showed the lowest glucose consumption and cell yield. At this temperature, CAM025 produced a high yield of EPS (Table 5.1). At -2°C, CAM025 may have had more energy to produce EPS as less was being used for production of cellular components than at higher incubation temperatures.

5.5.2. Regulation of EPS production at sub-optimal temperatures

Exopolysaccharide production is a major event in energetic terms and the rate of ATP utilization for its synthesis can be equivalent to 90% of that required for cell production (Linton et al. 1987). In continuous cultures of *Xanthomonas campestris* and mucoid *Pseudomonas aeruginosa*, the ATP demand for high specific rates of exopolysaccharide synthesis was a significant proportion of total

cellular ATP demand (Jarman and Pace 1984). Another critical aspect of EPS production involves the regulation of isoprenoid carrier lipids, which are involved in the biosynthesis of cell wall components such as lipopolysaccharides, teichoic acids and peptidoglycan as well as the biosynthesis of EPS (Sutherland 1982). The production of EPS can be enhanced at sub-optimal incubation temperature, where slower cell growth would result in a delayed demand for lipid carrier, allowing it to be used in EPS production rather than in the production of wall polymer (Sutherland 1982). In addition, in stationary phase when cell growth and cell wall synthesis has stopped, more isoprenoid carrier could be available for EPS synthesis. Growth and EPS production could be in competition for lipid carriers (Petry et al. 2000). Due to this competition, culture conditions resulting in reduced growth rates such as low temperature could increase EPS production (Looijesteijn and Hugenholtz 1999). In studies with lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* NIZO B40, results showed that growth was limited by more than the availability of lipid intermediates. Growth and EPS production did not only share the use of lipid intermediates, but were dependent on substrate uptake, availability of sugar nucleotides and energy supply. Non-growth associated ATP consumption was relatively high at low growth rates, where energy supply could also have been the limiting factor for EPS production. Optimal temperature for EPS production and growth were not the same for this strain (Looijesteijn and Hugenholtz 1999).

In the current study, EPS production occurred during stationary phase as demonstrated by increasing viscosity after exponential growth had ceased. In addition, increased EPS yield at -2°C and 10°C indicated EPS production was occurring in these cultures at suboptimal temperatures. Optical density measurements for strain CAM025 indicated a higher cell yield for cultures incubated at 10°C compared to those incubated at 20°C. These results also suggest that the optimal temperature (i.e., the temperature at which the growth rate was the fastest) for growth in strain CAM025 may be between 20°C and 10°C. Previous studies of sea ice isolates in the genus *Pseudoalteromonas* and closely related to CAM025 suggested that the optimum temperature for growth would be in the

20°C to 22°C range (Bowman 1998). Further work is necessary before the precise optimal temperatures for cell growth and EPS production are known for strain CAM025.

High growth yield at suboptimal temperature has been noted in other studies. In order to remain competitive and to compensate for low growth rates at temperatures below optimal, psychrotolerant strains may maximize or maintain their growth yields at low temperature (Bakermans and Nealson 2004, Harder and Veldkamp 1968). In mesophiles, the maximum growth yield generally occurs near to the optimum growth temperature and can remain constant over parts of the growth temperature range where growth rate is constant. In comparison, the dependence of growth yield on temperature and other physical and chemical parameters may be a trait exhibited in other psychrotolerant strains as a class, but as yet has not been fully investigated (Bakermans and Nealson 2004).

5.5.3. Implications for the sea ice

Temperature is the cardinal factor controlling the rate of growth when other factors such as nutrient status and available water are non-limiting (Ratkowsky et al. 1982). Sea ice is an inherently heterogeneous environment for microbes. In the formation of sea ice, as the fresh water freezes and thaws, concentrations of brine and dissolved organic carbon vary considerably (Priddle et al. 1996). Even when substrates are available, heterotrophic bacteria rarely experience optimal temperatures for growth, but *in situ* studies have shown resident microbial communities are abundant and active (Junge et al. 2004). The EPS produced by CAM025 has a high average molecular weight (Mancuso Nichols et al. 2005c) and may be providing protection against extreme conditions present in the sea ice.

In other Antarctic studies, a fungal species isolated from the Dry Valleys produced a high molecular weight EPS; authors suggested a cryoprotective role (Selbmann et al. 2002). A strain of *Pseudomonas* isolated from temperate soil was shown to increase production of EPS with high water holding capacity when exposed to desiccation (Roberson et al. 1993). Experiments with xanthan gum showed that at a concentration of 50% in freshwater, freezing point was depressed to –13°C

(Yoshida et al. 1990, Krembs et al. 2002). The latter authors showed that Arctic sea ice had a high EPS content and suggested it may have an important role in the protection of cells in high salinity (up to 15%) and low temperature (as low as -10°C) brine channels. The high molecular weight EPS produced by CAM025 may, therefore have a cryoprotective effect in the Antarctic sea ice environment.

In permanently cold waters, rates of bacterial growth and respiration may be limited by the availability of organic or inorganic substrates or by the interaction of both with temperature (Pomeroy and Wiebe 2001). Microbes may reach a no-growth condition only when brine pockets freeze in winter sea ice. The availability of liquid water is the ultimate limit for growth. Bacteria growing at sub-optimal temperatures need higher concentrations of nutrients before they are able to make use of them (Pomeroy and Wiebe 2001), therefore nutrients may accumulate because of low substrate affinity of heterotrophic bacteria. In the current study, CAM025 incubated at -2°C consumed only 14% of the available glucose and produced a high yield of EPS. Substrates present in the media other than glucose, such as peptone or yeast extract may have been used in preference to glucose for EPS production. Alternatively, at low temperatures, such as those found in sea ice, CAM025 may have a decreased ability to take up and utilize nutrients as suggested by other studies (Pomeroy and Wiebe 2001). In the heterogeneous milieu of sea ice, microbes such as CAM025 may produce EPS intermittently as nutrient concentrations increase to useable levels.

In continuous fermentation studies with the mesophile *Pseudomonas* NCIBI 11264, EPS production was influenced by media composition, temperature, pH and the growth rate of the organism. However, the polysaccharide varied little in overall composition irrespective of pH, temperature, nitrogen, carbon and phosphate content of the growth medium (Williams and Wimpenny 1978). Culture conditions generally did not affect the types of monosaccharides in an EPS produced by halophilic bacterium *Halomonas maura* (Arias et al. 2003). Monosaccharide analysis of the EPS produced by CAM025 at -2°C, 10°C and 20°C, revealed no differences in the types or percentages (of total EPS) of monosaccharides present in the polymer (data not shown). However, analyses

showed that there was a higher percentage of uronic acids in the EPS produced at -2°C and 10°C than at 20°C (Figure 5.5).

Crude chemical analysis of EPS produced by CAM025 showed that there was a higher percentage of uronic acids in the EPS produced at -2°C and 10°C than at 20°C (Figure 5.5). Monosaccharide analyses also showed a higher percentage of galacturonic acid in EPS produced by cultures incubated at –2°C relative to cultures incubated at 20°C. During each Austral summer, approximately four fifths of the sea ice surrounding Antarctic melts (Zwally et al. 1983), releasing dissolved and particulate material, living cells and aggregations of cells into the surrounding waters (Brierley and Thomas 2002). Arctic sea ice studies (Krembs and Engel 2001, Krembs et al. 2002) demonstrated that microbially produced neutrally buoyant EPS was carried large distances by prevailing under-ice currents and ice drifts. Studies in more temperate waters showed marine bacterial EPS production played a major role in the aggregate formation process (Biddanda 1986, Decho 1990). When released into the water column, a combination of biological, chemical and physical forces caused this colloidal material to form aggregates (Alldredge and Jackson 1995), which became centers of high heterotrophic microbiological activity (Kiorboe 2001). The availability of iron as a trace metal is of critical importance in the Southern Ocean where it is know to limit primary production (Scharek et al. 1997). Since 99% of dissolved iron in the ocean is bound to organic ligands (Rue and Bruland 1995), polyanionic EPS such as those produced by CAM025 may have an important role in the Antarctic marine environment. Microbial EPS may act to keep iron in solution and accessible for primary productivity (Geider 1999) or as the framework of sinking aggregates, transporting bound iron out of the euphotic zone (Wu et al. 2001).

5.6. Conclusions

CAM025 is a halotolerant, psychrotolerant sea ice isolate, belonging to the genus *Pseudoalteromonas*, which is known to abound in the sea ice microbial community. The enhanced production of a high molecular weight polyanionic EPS at sub-optimal incubation temperatures lends support to theories that EPS

may have a cryoprotective role in the high salinity, low temperature habitat of sea ice brine channels. The importance of the role of EPS in sea ice as well as in the Southern Ocean, where the availability of trace nutrients such as iron limits primary production and CO_2 sequestration, requires further attention.

5.7. ACKNOWLEDGEMENTS

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Chapter 6. *Olleya marilimosa* gen. nov., sp. nov., an exopolysaccharide producing marine bacterium from the family *Flavobacteriaceae*, isolated from the Southern Ocean

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The research within the original manuscript and this resulting Chapter was conducted by myself. Co-authors John Bowman and Jean Guezennec fulfilled supervisory roles.

6.1. ABSTRACT

A Gram-negative, aerobic, gliding, orange-yellow marine bacterium was isolated from particulate material sampled from the Southern Ocean. This strain produced an exopolysaccharide in liquid culture. 16S rRNA gene sequence analysis showed that this isolate was a member of the family *Flavobacteriaceae*, but represented a separate lineage. Major whole cell fatty acids included i15:1ω10c, i15:0, β-OH i15:0, a15:1ω10c, 15:0, α-OH i15:0. The DNA base compositions was 49 mol% G+C. Based on phylogenetic, phenotypic, chemotaxonomic and genotypic analyses, this novel bacterium was placed in a novel taxon as *Olleya marilimosa* gen. nov., sp. nov. with type strain CAM030^T (= ACAM 1065^T = CIP 108537^T). The GenBank accession number for the 16S rRNA gene sequence of strain CAM030^T is AY586527.

6.2. INTRODUCTION

The family *Flavobacteriaceae* (Bernardet et al. 2002) is one of the major branches of the Gram-negative phylum "*Bacteriodetes*" that has been known until recently as the *Cytophaga-Flexibacter-Bacteroides* (CFB) group (Garrity and Holt 2001). Within this family, 16S rRNA gene phylogenetic analyses have shown that many marine species cluster into a well-defined "marine clade," which dominates marine and marine-derived surface waters (Bowman and Nichols in press). In the world's oceans, members of the "marine clade" of the *Flavobacteriaceae* make a significant contribution to the materialization of organic matter (Kirchman 2002). Community structure studies of microbial assemblages in the Southern Ocean have shown that this group forms a substantial proportion of the heterotrophic microbial biomass (Simon et al. 1999).

Marine aggregates are ubiquitous and abundant in the world's oceans (Fowler and Knauer 1986) and consist of complex assemblages of zooplankton fecal pellets, phytoplankton and other material enriched in bacterial communities (Logan and Hunt 1987, Mueller-Niklas et al. 1994) dominated by members of the family *Flavobacteriaceae* (Kirchman 2002). As centers of high bacterial activity, marine aggregates are believed to have a major role in the downward transport of carbon (Kiorboe 2001). In the Ross Sea near Antarctica, concentrations of aggregates were greater than at most other locations in the oceans (Asper and Smith 2003) and aggregate sinking accounted for a significant proportion of transport of organic material to bottom waters and sediments. Exopolysaccharides (EPS) secreted by bacteria are among the polymeric substances that provide a network to hold these structures together (Flemming and Wingender 2001a).

The availability of iron (Fe^{+3}) as a trace metal is of critical importance in the Southern Ocean where it is known to limit primary production (Scharek et al. 1997). As much as 99% of dissolved iron in the ocean is bound to organic ligands (Rue and Bruland 1995). Results from a recent study indicated that the EPS produced by Antarctic bacterial isolate CAM030^T derived from Southern Ocean particulate material included uronic acids (Mancuso Nichols et al. 2005c). These

monosaccharide components are negatively charged at seawater pH, give the EPS a 'sticky' quality (Decho 1990, Sutherland 2001) and may influence the availability of trace metal such as iron. EPS similar to those produce by CAM030^T may be acting as ligands for cations such as iron and other trace metals in the Southern Ocean environment.

Phylogenetic analysis of CAM030^T showed that this bacterium belongs to the family *Flavobacteriaceae*, but represents a separate lineage (Mancuso Nichols et al. 2005c). In the current study, we provide results of chemotaxonomic, genomic and phenotypic studies that support the placement of this strain in a new genus, *Olleya marilimosa*, gen. nov., sp. nov. in the family *Flavobacteriaceae* with type strain CAM030^T.

6.3. Materials and Methods

6.3.1. Isolation of strain

Samples for isolation of bacteria were obtained during the November/December 2001 voyage of RSV *Aurora Australis.* CAM030^T was isolated from material sampled from the cod end of a plankton net (20 μm) trawled through the Southern Ocean at approximately: 65°32'06"S, 143°10'16"E, where the sea temperature was 4°C and salinity was 3.5%. Isolations were carried out according to methods described previously (Mancuso Nichols et al. 2005c).

6.3.2. Phenotypic analysis

Phenotypic methods used to characterize strain CAM030^T have been described elsewhere by Bowman et al. (1996, 1997b). Unless otherwise specified, marine agar (1 g yeast extract, Oxoid L21; 5 g bacteriological peptone, Oxoid L37; 32 g artificial sea salts, Sigma S9883; 15 g agar; 1000 ml distilled water) was used as a basal media and incubations were carried out at 20°C. Motility was tested using the hanging drop method and gliding motility was examined after growing the strain for 1-2 days at 12°C on 0.1X marine agar (solidified with 1% agar). After incubation, growth margins were observed using phase contrast microscopy (Bowman et al. 2003). Media used in testing for hydrolysis of starch, tyrosine,

xanthine, crystalline cellulose, esculin, elastin, and for utilization of uric acid were supplemented with 3.2% (w/v) artificial sea salts (Atlas 1993). DNA hydrolysis was tested by using DNase test agar (Oxoid, CM321). Lipase activity, Tween 80 and casein hydrolysis were tested as described previously (Smibert and Krieg 1994). Acid production from glucose was determined according to the method described by Leifson (Leifson 1963). Additional biochemical tests were carried out using API 20E, API 20NE and rapid ID 32A strips (bioMérieux, Marcy l'Etoile, France) according to the manufacturers instructions and as described previously (Bowman et al. 1996). For these tests, inoculating or suspension media contained 3.2% (w/v) artificial sea salts. API 20E and API 20NE strips were incubated at 20°C for 3 days, while rapid ID 32A strips were incubated for 24 hr at 20°C. The results of phenotypic test are given in the species description.

6.3.3. Phylogenetic analysis

The 16S rRNA gene sequence analysis of CAM030^T was carried out according to procedures described previously (Bowman et al. 1996, Mancuso Nichols et al. 2005c) The phylogenetic tree (Figure 6.1) included 16S rRNA gene sequences from *Flexibacter flexilis* ATCC 23079^T (M62794) and *Chlorobium limicola* UdG-6037 (AJ299414) as outgroups. The GenBank accession number for the 16S rRNA gene sequence of *Olleya marilimosa* CAM030^T is AY586527. Bootstrap analysis was performed with 500 resampled data sets by using the SEQBOOT and CONSENSE programs from PHYLIP (Felsenstein 1993). High molecular weight DNA for G+C content was extracted using the Marmur technique (Marmur and Doty 1962). DNA G+C content was determined by the thermal denaturation procedure using spectrophotometry (Sly et al. 1986, Bowman et al. 1998).

6.3.4. Chemotaxonomic analysis

Whole cell fatty acid analysis was performed on CAM030^T grown for four weeks at 12°C on marine agar. Extraction and analysis of whole cell fatty acids was carried out according to procedures described elsewhere (Mancuso Nichols et al. 2005c). Fatty acids are designated by the total number of carbon atoms:number of double bonds, followed by the position of the double bond from the terminal

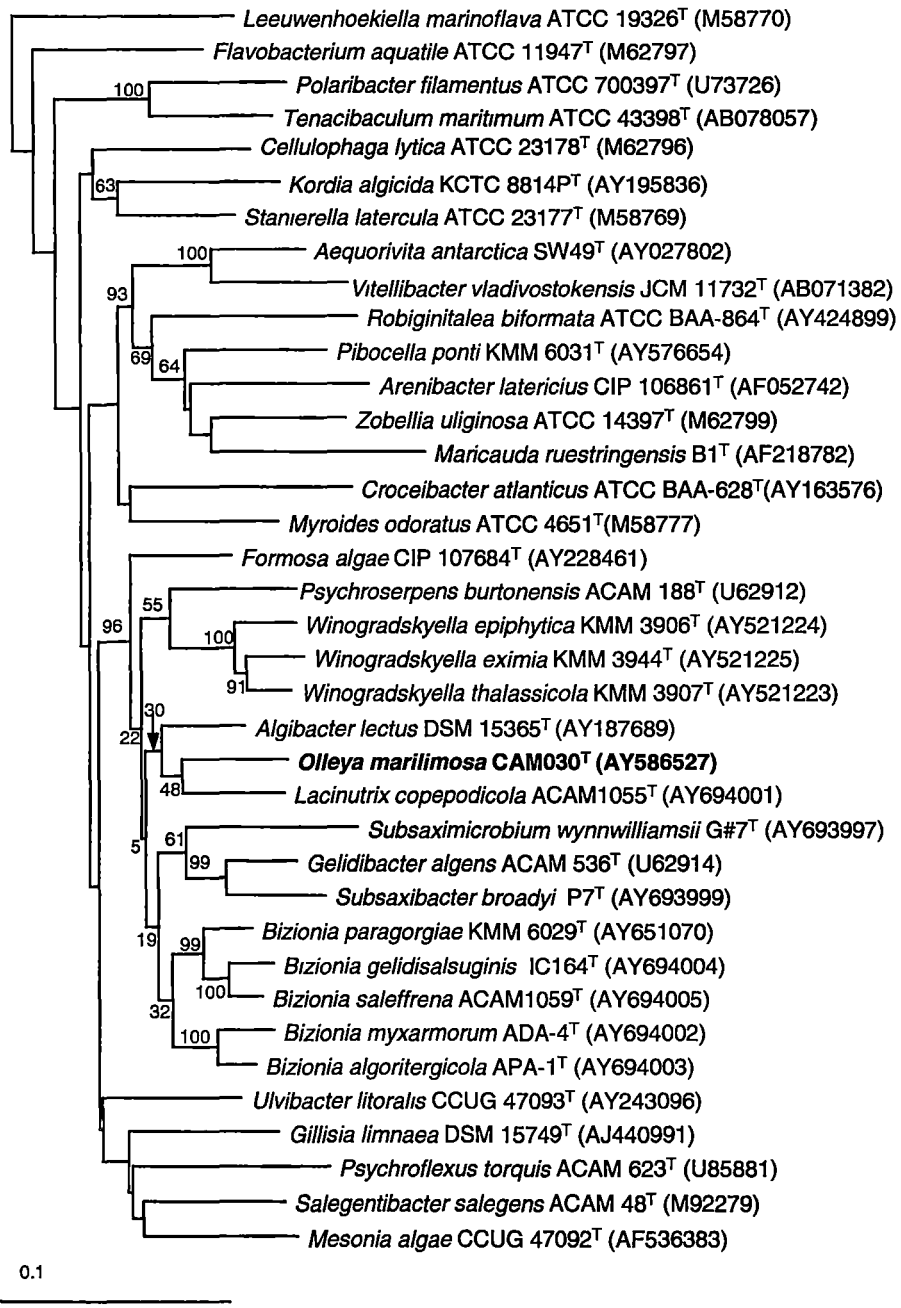

Figure 6.1. Phylogenetic relationship relationship of Antarctic marine bacterial isolate CAM030^T within the family *Flavobacteriaceae*. The tree is based on 16S rRNA gene sequences (positions 31-1470, *E coli* equivalent) and was created using maximum-likelihood distanced clustered by the neighbor-joining method. Bar: 0.1 changes per mean nucleotide position. Numbers in parentheses are GenBank nucleotide accession numbers. 16S rRNA gene sequences from *Flexibacter flexilis* ATCC 23079^T (M62794) and *Chlorobium limicola* UdG-6037 (AJ299414) were used as outgroups. Bar, 0.1 changes per mean nucleotide position.

(ω) end of the molecule. The suffixes c and t indicate *cis* and *trans* geometry and the prefixes i and a indicate iso and anteiso branching. The position of hydroxyl group (OH) may occur on the second (α) or third (β) carbon from the carboxyl end of the molecule.

6.4. Results and Discussion

16S rRNA gene sequence analysis showed that CAM030^T was distinct from all known cultured members of the family *Flavobacteriaceae*. *Lacinutrix copepodicola* ACAM 1055^T, *Bizionia saleffrena* ACAM1059^T, *Bizionia paragorgiae* KMM 6029^T and *Algibacter lectus* DSM 15365^T were the most closely related bacteria with sequence similarities of 94.0%, 94.1%, 94.2% and 94.5%, respectively (Figure 6.1). The low bootstrap support (<50%) for the 16S rRNA gene sequence of CAM030^T with other related members of the family *Flavobacteriaceae* further suggests that CAM030^T represents a discrete taxon. Characteristics used to differentiate CAM030^T from other closely related genera within this family are shown in Table 6.1. DNA G+C content analysis CAM030^T revealed a value of 49 mol%, which also suggests that CAM030^T is distinct from other related species (Table 6.1).

The major whole cell fatty acids present in CAM030^T were i15:1ω10c (22%), i15:0 (19%), β-OH i15:0 (10%), a15:1ω10c (8%), 15:0 (7%), and α-OH i15:0 (7%). The whole cell fatty acid profile of CAM030^T as compared to those of related genera is available as supplementary data (Table 6.2). Major fatty acids found in CAM030^T and found in other closely related genera, as well as in other members of the family *Flavobacteriacea,* are also listed in Table 6.2 (Bowman et al. 1998, Bowman et al. 2003). The predominance of branched saturated, branched monounsaturated and branched hydroxy fatty acids is a common characteristic in the *Flavobacteriaceae* (Bowman et al. 1998, Bowman et al. 2003, Nedashkovskaya et al. 2005b). It is interesting to note that for CAM030^T as well as for two closely related genera, *Algibacter* and *Lacinutrix*, there were few minor fatty acids with a chain length other than 15 carbons, with the exception of br16:1 (5%) and β-OH i17:0 (9%)

Table 6.1. Differential of *Olleya marilimosa* gen. nov., sp. nov. (CAM030^T) from related genera belonging to the family *Flavobacteriaceae*

Data from Bowman et al. (1997), Ivanova et al. (2004), Nedashkovskaya et al. (2004a), Nedashkovskaya et al. (2004b), Nedashkovskaya et al. (in press) and Bowman and Nichols (2005) and this study. YL, Yellow; OR, Orange; -, Negative; +, Positive; A, Aerobic; F, Facultatively anaerobic; V, Characteristics vary among species within this genus; ND, Not determined. All genera positive for catalase, negative for flexirubin pigments, growth at 37°C, production of indole and urease, degradation of crystalline cellulose.

Characteristic	**CAM030^T**	***Psychroserpens***	***Gelidibacter***	***Lacinutrix***	***Algibacter***	***Formosa***	***Winogradskyella***	***Bizionia***
Cell morphology	Rods with tapered ends	Ring shaped, helical or coiled cells	Rods	Straight or slightly curved rods	Rods	Slightly pointed rods	Rods	Rods
Pigment production	OR/YL	YL	YL/OR	YL	OR	YL	YL	YL
Gliding motility	+	-	+	-	+	+	+	-
Requirement of Na^+	+	+	V	+	+	-	+	+
Growth at 25°C	+	-	V	V	+	+	+	V
Growth at 30°C	+	-	-	-	+	+	+	V
Metabolism	A	A	A	A	F	A	A	A
Acid production from carbohydrates	+	-	V	-	+	+	V	-
Acid production from glucose	+	-	+	-	+	+	V	-
Production of								
DNase	-	-	V	-	-	ND	V	V
oxidase	+	-	-	-	+	-	+	+
β-galactosidase	-	ND	V	-	+	ND	-	-
Nitrate reduction	-	-	V	-	-	+	-	-
Carbohydrate utilization	+	-	+	+	+	+	V	-
Degradation of								
agar	-	-	-	-	+	-	+	-
starch	-	-	V	-	+	+	V	-
esculin	-	-	V	-	ND	ND	ND	-
casein	-	+	V	-	-	-	V	+
gelatin	+	V	V	+	+	+	+	+
H_2S production	-	-	-	-	-	-	V	+
G+C content (mol%)	49	27-29	37-42	37	31-33	34-35	35	38-45

found in *Lacinutrix* and *Algibacter* respectively. Variations in culture conditions can have a significant impact on the type and abundance of whole cell fatty acids. Presently, it is difficult to draw further conclusions from discrepancies in fatty acid profiles obtained from strains grown under dissimilar laboratory conditions.

Based upon the above information, it is proposed that CAM030^T represents a new genus and species in the family *Flavobacteriaceae*, *Olleya marilimosa* gen. nov., sp. nov..

6.4.1. Description of Olleya *gen. nov.*

Olleya (Ol.ley'. a N.L. fem. n. Olleya, named in honor of June Olley who has made significant contributions to the area of predictive microbiology).

Gram-negative rods, approximately 0.3 – 0.5 μm in width and 2.0 - 2.5 μm in length. Motile by gliding. Endospores are not formed. Cell mass is orange/yellow. Flexirubin pigments are absent. Strictly aerobic chemoheterotrophic. Produces catalase. Produces acid from carbohydrates. Major fatty acids include i15:1ω10c, i15:0, β-OH i15:0, a15:1ω10c,15:0, and α-OH i15:0. Phylogenetically, is a member of the family *Flavobacteriaceae*, class *Flavobacteria*, phylum "*Bacteriodetes*". The type species is *Olleya marilimosa* CAM030^T.

6.4.2. Description of Olleya marilimosa *sp. nov.*

mar i lim o' sa, L. gen. neut. n. *maris*, of the sea; L. adj. *limosus -a -um*, full of slime, slimy; N.L. fem. adj. *marilimosa*, of the sea and slimy. Description is as the genus description plus the following. When incubated on marine agar for one week at 20°C, CAM030^T formed orange/yellow, translucent colonies are 1-2 mm in diameter, circular, convex, with an entire edge and a butyrous consistency on marine agar. Colonies exhibit spreading margin on dilute agar and enhanced mucoid morphology when grown on marine agar supplemented with 3% glucose. Growth occurred in the pH range 5 to 9 and in the temperature range of 4°C – 30°C. No growth occurs at 37°C. Requires Na^+ or sea salts for growth. Growth occurs between 0.2 to 0.9 M NaCl with optimal growth occurring at

Table 6.2. Whole cell fatty acid profiles of CAM030T and related genera in the family *Flavobacteriaceae**

Fatty Acid	CAM030[T]	*Psychroserpens*[†]	*Gelidibacter*	*Lacinutrix*	*Algibacter*	*Formosa*	*Winogradskyella*	*Bizionia*
Saturated acids								
12.0						0.5		
13 0	tr[‡]		tr	tr				tr
14.0	tr	tr	tr-0.6[§]	tr		1.0		tr-0.8
15:0	7.3	10.0	2 4-5.3	3.7	13 4	27.2	1.2-7.9	2 3-4.3
16.0	1 2	tr	1.2-2.2	2.9		1 0		1.2-3.4
17.0						0.6		
18:0			0.5-1 2	-				tr-1.1
Branched saturated acids								
i13·0	tr	tr	tr	tr				tr-3.0
i14.0		tr	tr-2.8	1.4		tr	1.4-4.5	1.2-2.5
i15.0	18.8	10 0	3.4-8-8	19.9	12.5	13.1	6.7-25.6	3.1-13.2
a15:0	4.0	10 4	10.5-17.7	18.2	7.2	7.2	4.9-15 9	10.1-20.8
i16:0	tr	0.6	1.4-4.4	7.1		2.3	0.8-5.7	1 3-5.8
a17.0			1.5				tr-2.3	3.7
Cyclopropyl acids								
cy17.0						7 4	tr-2 4	
cy19·0						tr		
10me16:0							tr-6 3	
Monounsaturated acids								
15:1		17.5	2.7-4.2	1.2				1.3-2.3
15:1ω8c						1.0		
15:1ω6c						13.0	tr-6.5	
15·1ω6t	2 1				10.9			
16·1ω9c		0.7						
16 1ω7c	1.3	tr	4.3-9.5	2.8		3.3	4.2-6.1	2.6-6.3
16·1ω5c		6 9	tr-1.5	-				tr-0.8
17.1ω8c		1.2				2 2		
17.1ω6c		1.5	(3.7)				tr-1.9	1 9
18:1ω9c						0.5		
18:1ω7c	tr							
Branched monounsaturated acids								
br14.1			tr-1.7	0.7				tr-1.1
i14:1							tr-1.4	
i15:1					13 4	15 3	8.1-11 4	
a15.1							1 4-6.3	
i15:1ω10c	21 9	14 1	5.3-11.4	12 0				2.1-12.5
a15:1ω10c	7.9	8 4	11.8-16.6	14.2				5.8-14.0
br16:1			1.4-10.3	4.6		1.0	2.7-4 7	1.2-5.4
i16·1ω11c		9.1						
i16·1ω6c	0 9		7 7					
i17 1	tr		1.1-2 3			1.4		1.7-15.1
i17.1ω9c							tr-1.1	
i17·1ω7c		1.5	4.9					
a17.1	tr	tr	1.9-3.4			tr		1.5-3.6
Hydroxy acids								
β-OH 13:0	tr							
β-OH 14:0						1.1	tr-1.6	
β-OH 15:0	3 8		tr-1.5	tr			tr-2.5	tr-1.2
α-OH 15.0								
β-OH 16 0	tr		tr				tr-1.0	
α-OH i15.0	6 8							
β-OH i15:0	10.4		2 2-6.2	3 1	9.4		2 6-11.9	1 2-9.3
α-OH a15:0	1.7		0 3-10 6	10.6			3.3-1.0	tr-22.9
β-OH i16·0			4.1-12.2		2 7		3.2-18.1	1 6-8.5
α–OH i16.0	4.4						0.8-1 0	
β-OH i17·0	4.4		tr-3.1	2.3	9.1	tr	5.4-7 3	tr-4.3
β-OH a17·0			1.0-11.3	1.7				tr-2.9
α-OHi13:1	1 7							
β-OH i16:1			0.8					
β-OH a17:1			tr					
unknown							3.7-5.6	

* Data reported as percentages of total fatty acids

† Data for *Psychroserpens burtonensis* ACAM188[T] from Bowman *et al.* (1997), for *Gelidibacter algens* ACAM 536[T], *Gelidibacter mesophilus* 2SM29[T], *Gelidibacter gilvus* ICI58[T], *Gelidibacter salicanalis* ICI62[T], *Lacinutrix copepodicola* DJ3[T], *Bizionia salifrenus* HDF[T], *Bizionia gelidisalsuginis* ICI164[T], *Bizionia algoritergicola* APA-1[T], *Bizionia myxarmorum* ADA-4[T], from Bowman and Nichols (in press), for *Bizionia paragorgae* KMM 6029[T] from Nedashkovskaya *et al.* (2005b), for *Algibacter lectus* KMM 30902[T] from Nedashkovskaya *et al.* (2004), for *Formosa algae* KMM 3553T from Ivanova *et al.* (2004), for *Winograskyella* thalassicola KMM 3907[T], *Winograskyella epiphytica* KMM 3906[T], *Winogradskyella eximia* KMM 3944[T] from Nedashkovskaya *et al.* (2005a) and this study.

‡ tr, less than 0.5% of total fatty acids.

approximately 0.2 to 0.5 M NaCl. Requires yeast extract or peptone for growth. Produces acid from glucose, assimilates a range of carbohydrates, but does not reduce nitrate to nitrite or produce H_2S. Indole, DNAse, β-galactosidase, lipase, urease and acetoin (Vogues-Proskauer reaction) were not produced, but oxidase and catalase were formed. Tween 80, elastin, gelatin and tyrosine were degraded, but agar, starch, esculin, casein, cellulose, and xanthine were not. Citrate was utilized as a sole carbon source; uric acid was not. Glucose, maltose and mannose were assimilated; arabinose, mannitol, D-gluconate, capric acid, adipic acid, malate and trisodium citrate were not. Tests for β-N-acetyl-glucosaminidase, alkaline phosphatase, arginine arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, and serine arylamidase were positive. Tests for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate, α-glucosidase, β-glucosidase, α-arabinosidase, β-glucuronidase, glutamic acid decarboxylase, α-fucosidase, proline arylamidase and pyroglutamic acid arylamidase were negative. The G+C content of the DNA is 49 mol%. Type strain, isolated from Southern Ocean particulate material is CAM030^T = ACAM 1065^T = CIP 108537^T

6.5. Acknowledgements

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Chapter 7. Preliminary investigations into the uptake of copper and cadmium by an Antarctic sea ice bacterial exopolysaccharide

Chapter 7. Preliminary investigations into the uptake of copper and cadmium by an Antarctic sea ice bacterial exopolysaccharide

7.1. Abstract

A sea ice bacterium isolated from Antarctica produced an exopolysaccharide with a high molecular weight (5700 kDa) and high viscosity (5000 cm^3/g at 0.1 mg/ml). Previous studies showed that the chemical structure of this EPS included uronic acid and sulfate groups. The copper and cadmium binding ability of the purified EPS was investigated and preliminary results showed that the EPS had a high affinity for both copper (23 mmol/g EPS) and cadmium (7 mmol/g EPS). This finding is one of the first steps in assessing the ecological role and biotechnological potential of the polysaccharides.

7.2. Introduction

Microbially produced exopolymers are abundant in aquatic systems, where they surround microbial cells in aggregates, in biofilms on surfaces and in sediments (Decho 1990). Exopolymers are excreted by bacteria as loose slime or as tightly bound capsular material. Extracellular polymeric substances enhance survival and change environmental conditions around the cell by altering the physico-chemical micro-environment (Costerton 1974). Compared to the lipid, protein and nucleic acid content, high molecular weight polysaccharides are the most abundant macromolecules in the exopolymers and make up 40-95% of the extracellular material (Flemming and Wingender 2001b).

Several studies have noted high heavy metal binding affinities for microbial exopolymers in habitats such as sediments, biofilms, sewage sludge flocs, marine and estuarine waters (Mittelman and Geesey 1985, Spaeth et al. 1998, Wuertz et al. 2000, Batley and Gardner 1978). Cellular proteins are important in this

process, as well as polysaccharide secretions, which are often high in functional groups such as the carboxyl groups of uronic acids. In the marine environment, uronic acids are common constituents of microbial exopolysaccharides (EPS, Kennedy and Sutherland 1987). At pH values greater than 7, such as those found in the marine environment, the presence of uronic acids and sulfate functional groups in the polymer increases the anionic character of polysaccharides (Sutherland 1990, Sutherland 2001). Acidic carboxyl or sulfate functional groups as well as hydroxyl groups, which are abundant in polysaccharides, can serve as binding sites for metal ions (Rendleman 1978a).

Bacteria isolated from deep-sea hydrothermal vent environments are characterized by tolerance to high pressure, temperature and dissolved sulfides and heavy metals. In one study, most bacteria isolated from polychaete annelids dwelling near the vents showed high resistance to cadmium, zinc, arsenic, silver and copper (Jeanthon and Prieur 1990). Among these isolates were strains shown to produce EPS with a strong ability to bind heavy metals (Loaec et al. 1997, Loaec et al. 1998). The authors attributed this ability to several qualities of the EPS including conformation of the polysaccharide network and the presence of hydroxyl groups, pyruvated mannose, sulfate esters, and a high uronic acid content. EPS such as this one have potential commercial applications such as in detoxification of heavy metal polluted waters and waste water treatment (Loaec et al. 1997).

A marine bacterium (strain CAM025), isolated from Antarctic sea ice belonging to the family *Pseudomonas* produced an EPS in laboratory culture. When purified, the EPS was shown to be of a high molecular weight (5700 kDa); the crude chemical composition included approximately 20% uronic acids and 5% sulfate (g/g EPS, Mancuso Nichols et al. 2004). The sea ice habitat is characterized by environmental extremes. In brine channels, salinity can reach 209 ppt, temperatures drop as low as –20°C and microbes have been found to thrive (Junge et al. 2004). Previous studies suggested EPS may provide some cryoprotection to bacteria living there (Krembs et al. 2002, Mancuso Nichols et al. 2005a). The availability of dissolved iron limits primary production in the Southern Ocean, where melting sea ice releases microbially produced EPS (Krembs and Engel

2001). Studies have shown that in this environment, most dissolved iron is sequestered by organic ligands with a high affinity for iron (Rue and Bruland 1995). Strain CAM025 belongs to a group of bacteria known to be common inhabitants of sea ice and Southern Ocean particulate material (Bowman et al. 1997a). EPS produced by these bacteria may have an important role in Southern Ocean and the global carbon cycle. This study was conducted to investigate the ability of EPS produced by strain CAM025 to bind heavy metals cadmium and copper as a first step in assessing the ecological role as well as the biotechnological potential of this EPS.

7.3. Materials and Methods

7.3.1. Growth of bacteria

7.3.1.1. Cadmium and copper tolerance

Marine agar (1 g yeast extract, Oxoid L21; 5 g bacteriological peptone, Oxoid L37; 32 g artificial sea salts, Sigma S9883; 4.76 g HEPES, Sigma H7637; 15 g agar and 1000 ml distilled water) was prepared and either $Cd(NO_3)_2 \cdot 4H_2O$ (Cat # 24,751-0, purity 98%, Aldrich Chemical Company Inc, Milwaukee, USA) or $CuCl_2 \cdot 2H_2O$ (Cat #D3247, Ajax Chemical, Sydney, Australia) was added for a final (Ca^{2+}) or (Cu^{2+}) concentration of 20 mg/L or 40 mg/L, respectively. Media was autoclaved without prior pH adjustment.

7.3.1.2. Batch cultures for EPS production

The bacterium CAM025 was grown in liquid culture at 0°C in broth composed of 1 g yeast extract, Oxoid L21; 5 g bacteriological peptone, Oxoid L37; 34 g artificial sea salts, Sigma S9883; 4.76 g HEPES, Sigma H7637; and 1000 ml distilled water. The pH of the broth was adjusted to 8 prior to autoclaving. A glucose solution was prepared and autoclaved separately before being combined with the above media for a final concentration of 3% glucose (w/v). One litre baffled flasks containing 500 ml of the above media were inoculated with 50 ml of the exponentially growing batch cultures of the bacterial isolate. Flasks were incubated for 2 weeks in an oscillating water bath (Ratek Pty Ltd, NSW, Australia) fitted with refrigeration units that cooled and circulated antifreeze

liquid at 0°C. The purity of the culture was checked by subculturing onto a MA+Glu plate and a 10 ml aliquot was removed to measure pH.

7.3.2. Isolation and purification of EPS

Cultures were diluted with three volumes of sterile artificial sea salts in distilled water (3.5% w/v, pH 8). Diluted broth was centrifuged at 30,000 g for 2 hr at 4°C. The cell pellet was frozen, freeze-dried and weighed. The supernatants were diafiltered with 10 volumes of sterile distilled water (pH 8) using a VivaFlow 200 ultrafiltration unit (Sartorius, Victoria, Australia) fitted with a 100,000 MWCO PES membrane. Ultrafiltration was performed at room temperature. The EPS solution was concentrated, frozen and freeze-dried. Protein content of purified EPS was measured according to the method described by Bradford (1976).

7.3.3. FT-IR Spectroscopy

Pellets for infrared analysis were obtained by grinding a mixture of 2 mg polysaccharide with 200 mg dry KBr, followed by pressing the mixture into a 16 mm diameter mold. The Fourier transform-infrared (FT-IR) spectra were recorded on a Bruker Vector 22 instrument (NSW, Australia) with a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} region. A qualitative estimate of EPS purity was determined by comparison of FT-IR spectra of EPS purified by ultrafiltration with spectra of EPS purified by as previous described (Mancuso Nichols et al. 2004).

7.3.4. Molecular weight and reduced viscosity determination

The molecular weight and reduced viscosity of the EPS purified by pressure filtration, ethanol precipitation and dialysis was determined by size exclusion chromatography (SEC, Busnel et al. 1995, Degoulet et al. 1995). The eluant was a 0.1 M $NaNO_3$ in water with 200 mg/L sodium azide added. The analyses were carried out at room temperature. An isocratic pump (Spectra Physics P100, Thermo Separations, Bordeaux, France) with a flow rate of 1 ml/min and a TSK gel PW6000 column (Toyo Haas, Frankfurt, Germany) was used (60 cm x 0.7 cm) with pectin as a standard. A differential refractometer (Shodex RI71, Shodex, Tokyo, Japan) on-line multiangle light scattering and viscosity detection described elsewhere (Busnel et al. 1995) were employed.

The polydispersity (I) is the ratio of the weight average molecular weight (M_w) to the molecular weight based on the number of molecules (M_n), relative to the pectin standard ($M_w / M_n = I$). Polydispersity gives an indication of the molecular weight distribution or range of size homogeneity within each polymer. As polydispersity increases, there is a larger difference between the sizes of the largest molecules and the smallest molecules.

7.3.5. Metal binding experiments

Purified exopolymer was dissolved in MilliQ water (18.2 MΩ/cm). Initial concentrations of 1mg/ml were used according to protocols described by Laoec et al (1997, 1998). Later this concentration was reduced to 0.01 mg/ml when problems in the methodology were attributed to the high viscosity of the polysaccharide in solution. Metals tested were cadmium and copper. Solutions of copper were prepared from $Cu(NO_3)_2 \cdot 2.5H_2O$ (Cat # 22,339-5, purity 98%, Aldrich Chemical Company, Inc, Milwaukee, USA). Solutions of cadmium were prepared from $Cd(NO_3)_2 \cdot 4H_2O$ (Cat # 24,751-0, purity 98%, Aldrich Chemical Company Inc, Milwaukee, USA). Reaction solutions were prepared in duplicate by adding 30 µl of a 1 mg/ml solution of EPS to a 15 ml polypropylene tube containing milliQ water and metal solution for a final concentration of 10 mg/L EPS and metals in the range of 10 to 300 mg/L. The total sample volume was 3 ml. Samples were mixed thoroughly. The pH of solutions prior to incubation was 4.2 for copper and 5.5 for cadmium.

Samples were incubated at 20°C and shaken at 200 rpm for 3 hrs. The sample was transferred from the original sample tube to an Amico Ultra-4 Centrifigal Filter device (Cat # UFC8-030-96, Millipore, Sydney, Australia). These devices were fitted with a low binding regenerated cellulose membrane filter with a 30,000 nominal molecular weight cut off. Samples were spun at 4000 g for 15 minutes in a fixed angle centrifuge (Sorvall, Auckland, NZ). The filter unit was removed and the filtrate was acidified by adding 300 µl of a 10% nitric acid solution (v/v, Merck Pty. Ltd., Victoria, Australia). The final pH of solutions was approximately 0.9. Metal concentrations in the filtrate were determined by atomic absorption spectrometry.

7.3.6. Atomic absorption spectrometry

Samples were analysed on an Avanta Σ Instrument (GBC Scientific Equipment Pty. Ltd., Victoria, Australia) with air (300-400 kPa, 10 L/min) as the oxidant and acetylene (55-96 kPa, 2 L/min) as the fuel. Lamp current was set at 3.0 mA and wavelengths of 249.2 nm (3-370 mg/L) and 327.4 nm (0-10 mg/L) were used for copper and 228.8 nm (0-1.8 mg/L) and 326.1 nm (3-800 mg/L) for cadmium. All measurements had background correction. Standard curves were linear at all metal concentrations analysed with these wavelengths.

7.4. RESULTS

CAM025 showed enhanced growth with mucoid morphology after 2 weeks incubation at 15°C on solid media amended with glucose and either cadmium or copper at concentrations of 20 mg/L and 40 mg/L respectively. The yield of purified EPS harvested from batch cultures after 2 weeks incubation at 0°C, was 343 mg/L. The protein content was approximately 2% of the total EPS weight. This is comparable to results from previous studies (Table 7.1), in which the EPS was purified by pressure filtration, cold ethanol precipitation and dialysis for 48 hr against distilled water (Mancuso Nichols et al. 2004). These results indicate that the EPS purified by ultrafiltration contained no additional cellular material, as indicated by the low protein content.

Table 7.1 Crude chemical composition of EPS produced by sea ice bacterium, CAM025

Neutral Sugars	Uronic Acids	Protein	Sulfates
74*	22	2	5

* % (g/100g)

FT-IR spectra of the EPS showed a broad O-H stretching band above 3000 cm^{-1} and intense absorptions between 1650 and 1050 cm^{-1} characteristic of polysaccharides. Weak absorbances at approximately 1730 cm^{-1} and 1230-1250

cm^{-1} indicated the presence of carboxyl and sulfate groups respectively (Lijour et al. 1994). These spectra were very similar to spectra from analysis of EPS from the same strain purified by pressure filtration, cold ethanol precipitation and dialysis (Mancuso Nichols et al. 2004).

Molecular weight and viscosity data are presented in Table 7.2. The purified EPS produced by CAM025 was found to have a molecular weight of 5700 kDa, with a polydispersity of 2.5. At a concentration of 0.1 mg/ml, the viscosity was 5000 cm^3/g.

Table 7.2. Viscosity and molecular weight of EPS produced by sea ice bacterium, CAM025

Conc (mg/ml)	Viscosity (cm^3/g)	Molecular weight (kD)	I_p (polydispersity)
0.1	5000	5700	2.5

In studies to investigate metal binding of the CAM025 EPS, a concentration of 0.1% EPS in water (1 mg/ml) was used initially according to the method of Loaec et al. (1997). However, the removal of the EPS bound metal from the solution prior to measurement of the equilibrium metal concentration was not possible by centrifugation due to the high viscosity of the solution. A 100-fold dilution of the EPS yielded a solution of sufficiently low viscosity to proceed.

The uptake of copper and cadmium by EPS produced by CAM025 are presented as mass of metal (mg) per gram EPS (Figure 7.1). An increase in initial copper concentration from 4 to 100 mg/L resulted in a forty fold increased uptake from 33 to 1347 mg copper/g EPS. Uptake of copper did not increase substantially at higher metal concentrations of 250 and 300 mg/L. This uptake is approximately

23 mmoles copper/g EPS, on a molar basis.

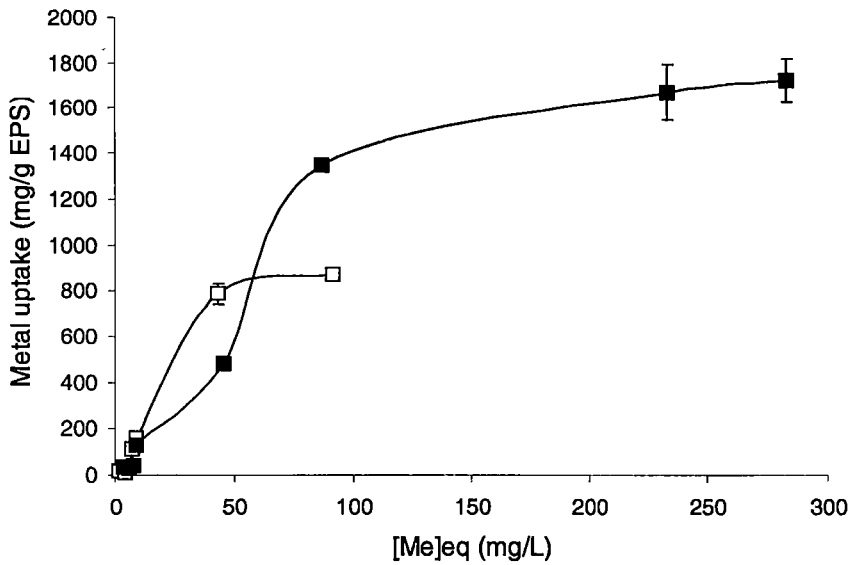

Figure 7.1. Uptake of metal by purified exopolysaccharide produced by sea ice bacterium CAM025 measured by atomic adsorption spectroscopy (metal uptake versus equilibrium metal concentration). Open symbols denote cadmium uptake and closed symbols denote copper uptake.

An increase in initial cadmium concentration from 2 mg/L to 50 mg/L resulted in an increase of metal uptake from 16 to 790 mg cadmium/g EPS (Figure 7.1). Uptake did not increase substantially when initial metal concentration was increased to 100 mg/L. On a molar basis, this uptake is equivalent to approximately 7 mmoles cadmium/g EPS.

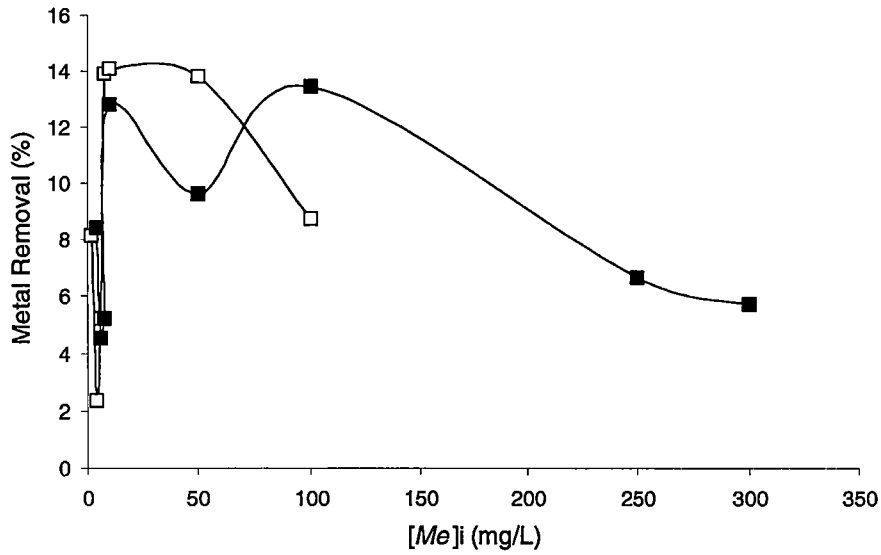

Figure 7.2. Metal removal by purified exopolysaccharide produced by sea ice bacterium CAM025 expressed as a percentage of initial metal concentration. Open symbols denote cadmium uptake and closed symbols denote copper uptake.

Metal removal efficiency, expressed as percentage of metal removed relative to initial metal concentration up to 100 mg/L cadmium and up to 300 mg/L copper, is presented in Figure 7.2. Maximum metal removal was reached (13%, copper; 14%, cadmium) at a metal concentration of approximately 10 mg/L for both metals and this coincides with the concentration of EPS (10 mg/L) present in the test solutions.

7.5. Discussion

7.5.1. Growth of strain CAM025

Growth of strain CAM025 on solid marine agar amended with copper and cadmium demonstrated that these heavy metals were not toxic to this strain at the concentrations tested. Enhanced mucoid colony morphology relative to growth of strain CAM025 on marine agar without added cadmium or copper indicated EPS

production may have occurred in response to exposure to the metal. In cultures of *Klebsiella aerogenes*, a noncapsulated mutant showed decreased survival compared to a capsulated strain when exposed to copper and cadmium concentrations of 10 mg/L in liquid media (Bitton and Friehofer 1978). When exposed to high levels of cadmium (250 mg/L), a strain of *Pseudomonas* isolated from soil, produced an EPS that accumulated cadmium and reduced soluble cadmium by 11% (Roane et al. 2001). The authors suggested that metal binding to exopolymers may have decreased the toxicity of the metal towards the bacteria.

7.5.2. Ultrafiltration

Ultrafiltration has been shown to be a rapid and effective method for the isolation and purification of EPS from bacterial cultures (Bergmaier et al. 2001). In this study, EPS produced in liquid culture was purified by centrifugation followed by an ultrafiltration step that included 10 volume exchanges with distilled water. The resulting polysaccharide had a low protein content (2%, w/w), indicating that cellular material had been largely removed. The FT-IR spectra were similar to those obtained after a much longer and time consuming purification protocol that involved pressure filtration through a range of cellulose nitrate filters of decreasing pore size followed by ethanol precipitation, washing with ethanol, freeze-drying, dialysis and refreeze-drying.

This procedure was further complicated when the viscosity of the polymer was high, such as in the case of the CAM025 polymer. A high viscosity polymer requires dilution early in the protocol, to facilitate centrifugation and pressure filtration. This dilution step increased the volume of the original culture broth significantly and subsequent processing time to prepare the dried and purified polymer. Harvesting of purified EPS from a 500 ml culture required approximately one day, not including freeze-drying of the final product, whereas the process involving ethanol precipitation required at least one week or more before the purified EPS was obtained. In the choice of ultrafiltration unit, previous knowledge of the molecular weight of the EPS allowed selection of a filter (100,000 MWCO) that would retain the EPS while removing salts and other substance of lower molecular weight to be separated.

7.5.3. Molecular weight and viscosity

The CAM025 EPS was a high molecular weight polymer with a low polydispersity (Table 7.1). The viscosity of a dilute solution of polysaccharide (0.1 mg/L) was also high at 5000 cm^3/g. An EPS produced by the hydrothermal vent bacterium *Alteromonas macleodii* subsp. *fijiensis* (Raguénès et al. 1996) was reported to have a viscosity of 2600 cm^3/g and this was considered high (Rougeaux et al. 1996). The structure and properties of EPS are influenced by the length of the polymer chain, that is, the molecular weight (Christensen 1999). As the length of the polymer increases, there is a greater opportunity for complex entanglement the chains and intramolecular associations, and these contribute to the tertiary structure and physical behavior of the polymer (Sutherland 1994). The viscosity of the polysaccharides is therefore related to its molecular weight.

In a study by Bamberg et al (2004), the chemical addition of sulfate groups increased the molecular weight and viscosity of an EPS from *Klebsiella oxytoca*. The reagent used in the modification reacted with free hydroxyl groups present and the authors suspected that the resulting cross-linking caused the increase in viscosity. The ability of the EPS to bind cadmium also increased substantially since the higher sulfate content increased the net negative charge of the polymer. The EPS examined in the current study consisted of 20% uronic acids and 5% sulfate (g/100 g EPS, Table 7.1) and it would be negatively charged in solutions with a pH greater than approximately 3.5. The presence of sulfate groups and carboxyl groups of uronic acids are extremely important in the ability of an EPS to bind metals (Volesky and Holan 1995).

7.5.4. Metal binding

Metal binding studies with the EPS produced by CAM025 showed an uptake of 1347 mg copper/g EPS (23 mmol/g EPS) and 790 mg cadmium/g EPS (7 mmol/g EPS) on a molar basis. Loaec et al (1997) reported an uptake of 125 mg cadmium/g EPS (1.12 mmol/g EPS) produced by the hydrothermal vent isolate, *Alteromonas macleodii* subsp *fijiensis*. The purified EPS produced by a freshwater sediment bacterium was shown to bind 253 μmol copper/g EPS (Mittelman and

Geesey 1985). The preliminary results presented for binding of cadmium and copper by the CAM025 EPS appear to be higher than values from previous studies.

The percentage of cadmium removed by EPS at a concentration of 10 mg EPS/L ranged from 8% of the initial metal in solution (2 mg Cd/L) to 9% (100 mg Cd/L) with a maximum of 14% cadmium removed (8-50 mg Cd/L). The percentage of copper removed by EPS at a concentration of 10 mg EPS/L ranged from 8% (4 mg Cu/L) to 6 % (300 mg Cu/L) with a maximum of 13% copper removed (10 mg Cu/L and 100 mg Cu/L). These values are low compared to other studies that report a 90% removal of cadmium at polysaccharide concentrations of 100 mg/L and initial cadmium concentration of 100 mg Cd/L. The low concentrations of polymer used in this study, warranted the use of correspondingly low metal concentrations. Impurities in copper and cadmium salts as well as in the purified polymer may have taken up binding sites on the EPS but impurities were not being measured by atomic adsorption spectrometry. In a similar study in which sensitive multielemental analysis was used to quantify metal concentrations in binding experiments with highly purified polymer, a variety of cations remained associated with the polymer (Mittelman and Geesey 1985).

The assumption that one metal is bound to one binding site on the EPS is an underestimation of the potential binding ability of the EPS, which in solution becomes a complex three-dimensional gel. The simple models used in these studies, such as the Langmuir isotherm, do not incorporate the effects of external environmental factors. Isotherms generated using these models may be irregular since both the complexity of the metal and that of the polysaccharide are not taken into account (Volesky and Holan 1995).

7.5.5. Factors affecting metal binding

The binding of metal by complex polysaccharides is determined by many factors, including the spacing of the ionized groups, such as the carboxyl group of uronic acids or the sulfate functional groups, along the polymer chain and how the environment around these ionized groups effects the strength of its charge

(Rendleman 1978a). Other groups in the polysaccharide such as hydroxyl groups may also be important depending on their location and orientation with respect to the metal. The metal cation may bind to more than one anionic donor group located on the same polymer or on separate chains. The flow characteristics or viscosity of the polysaccharide can also be important. Finally, the steric fit of the metal within the frame work of the polymer gel network also has an affect on the polysaccharides affinity for metals (Rendleman 1978b).

7.5.6. Solubility of metals and pH

The pH of the solution is one of the most important factors affecting metal binding to the EPS. In a study examining metal binding to a hydrothermal vent bacterially derived EPS, uptake increased at higher pH for all metals tested (lead, cadmium and zinc, Loaec et al. 1998). At pH lower than 4, binding was reduced because of competition between metal and H^+ for the carboxylic site, above pH 7 free metal was decreased because metal hydroxides were formed, removing metal from the system (Loaec et al. 1998). A similar trend was observed in copper binding studies involving a freshwater sediment bacterial EPS (Mittelman and Geesey 1985). The authors suggested that an increase in pH apparently reduced the concentration of copper species capable of binding to EPS, while a decrease in concentration of H^+ ions competing with copper at the binding site resulted in a more stable interaction (higher affinity) between the reactive copper species and the exopolymer.

Copper solubility decreases rapidly between pH 6 and 8. In the current study, the pH of the copper-EPS solutions was approximately 4.2. Cadmium solubility decreases rapidly between pH 8 and 11. In the current study, the pH of the cadmium-EPS solutions was 5.5. At this pH, both cadmium and copper are soluble while uronic acids would be anionically charged and available for binding to metal in solution. In a study examining metal removal by capsulated cells of *Klebsiella aerogenes*, the authors noted that more soluble metals generally displayed the lowest removals and less soluble metal displayed the highest removals (Brown and Lester 1982). In order for metal binding to occur, the ligand must first overcome the hold the solvent has on the metal cation. Once this has occurred, the ionic bond between the metal cation and the ligand anion occurs

quickly (Rendleman 1978a). In the current study, copper appeared to show a higher affinity for the CAM025 EPS than cadmium. Copper is less soluble at lower pH than cadmium, consistent with a higher affinity for copper relative to cadmium. Copper readily binds to organic matter and this makes it one of the most toxic metals in aquatic environments (Mittelman and Geesey 1985), even compared to cadmium (Bitton and Friehofer 1978). The affinity of these dissolved metals for exopolymer binding sites has many environmental implications.

A study by Cowen and Silver (1984) provided evidence that iron and manganese was being deposited onto bacteria associated with rapidly sinking particles of marine snow in the Pacific Ocean. Below 100 m, bacteria were found with extracellular capsules containing metal precipitates and the frequency of these capsules increased with depth. The capsular metal deposits appeared to contribute a major portion of the weakly bound fraction of the particulate iron flux. The authors suggested the bacterial activity in the microenvironment of the marine snow particles was affecting the solubility of iron, since at the high pH of seawater, it would otherwise precipitate out of solution. Microscopic and laboratory studies have shown that bacterially produced EPS have a major role in marine snow formation (Biddanda 1986) and may have affected the solubility and binding of iron to these bacteria and particles. In the subarctic Pacific Ocean, Maldonado and Price (1999) showed that heterotrophic bacteria play a significant role in dissolved iron uptake and that the iron bound to strong organic ligands, the most predominant form of iron in the sea, is available to phytoplankton in these environments. In the Southern Ocean, iron limits primary production and is bound to organic ligands with a high affinity for iron (Rue and Bruland 1995). In this environment microbially produced EPS, such as the one examined in this study, may play an important role in the availability of iron and primary productivity.

7.6. Conclusions

EPS from CAM025 is a complex, high molecular weight polysaccharide that displays a very high viscosity at low concentration in aqueous solution. This EPS appears to have a high binding capacity for copper and cadmium. Further

structural elucidation will allow a better understanding of the mechanism of this affinity, and will also gauge its potential commercial value as a bioadsorbent for these and other heavy metals. Future research should involve experiments to assess the affinity for other metals, especially dissolved iron at low concentrations such as those found in the Southern Ocean. This will provide insight into the ecological role of EPS in the Antarctic marine environment.

7.7. ACKNOWLEDGEMENTS

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Chapter 8. Conclusions

Chapter 8. Conclusions

Exopolysaccharides (EPS) are high molecular weight carbohydrate polymers that make up a substantial component of the extracellular polymers surrounding most microbial cells in the marine environment. EPS comprise a large fraction of the reduced carbon reservoir in the ocean and enhance the survival of marine bacteria by influencing the physico-chemical environment around the cell.

EPS may have an important role in the Antarctic marine environment, possibly acting as ligands for trace metal nutrients such as iron or providing cryoprotection for growth at low temperature and high salinity. Ten bacterial strains (CAM003, CAM005, CAM006, CAM015, CAM023, CAM025, CAM030, CAM036, CAM064, CAM090), isolated from Southern Ocean particulate material or from sea ice, were characterized. Whole cell fatty acid profiles and 16S rRNA gene sequences showed that the isolates included representatives of two families: the *Altermonadaceae* including strains belonging to the genera *Pseudoalteromonas* and *Shewanella*; and the *Flavobacteriaceae* including strains belonging to the genera *Polaribacter* and *Flavobacterium.* In addition, there was also one strain, which was a member of the family *Flavobacteriaceae*, but represented a separate lineage. Based on phylogenetic, phenotypic, chemotaxonomic and genotypic analyses, this bacterium was placed in a novel taxon as *Olleya marilimosa* gen. nov., sp. nov. type strain CAM030^T.

The ten isolates were members of the phyla *"Gammaproteobacteria"* and *Cytophaga-Flexibacter-Bacteroides*, the taxonomic groups that have been shown to dominate polar sea ice and seawater microbial communities. The ten strains were grown in batch cultures containing marine salts media. The EPS produced were extracted from the culture broth, purified and characterized using a range of methods. Information on crude chemical composition, monosaccharide ratios and molecular weight data revealed that these EPS were diverse. EPS produced by two closely related *Pseudoalteromonas* isolates (CAM025 and CAM036), one from the sea ice and the other from particulate material were examined more closely. Although there were many similarities in these two EPS, such as proportion of

uronic acids in crude composition, there were also differences, including the composition of various monosaccharides present in each polymer, their relative abundances and functional groups. Most of the ten EPS examined in this study contained charged uronic acid residues; several contained sulfate groups. Molecular weights also varied significantly between these ten EPS, which seems to reflect the biodiversity present in the Antarctic marine environment.

The effect of temperature on the growth and EPS production of isolate CAM025 was also studied. Previous studies showed that this isolate was a member of the genus *Pseudoalteromonas*, and therefore belonged to a group found to be abundant in sea ice by culture dependent and independent techniques. This bacterium displayed psychrotolerant growth (at –2 to 30°C) in the laboratory. Batch cultures were grown at –2°C, 10°C and 20°C and cell number, optical density, pH, glucose concentration and viscosity were monitored. The yield of EPS at –2°C and 10°C was 30 times higher than at 20°C, which was the optimum growth temperature for many psychrotolerant strains. EPS may have a cryoprotective role in brine channels of sea ice, where extremes of high salinity and low temperature impose pressures on microbial growth and survival. EPS produced at –2°C and 10°C had a higher uronic acid content than that produced at 20°C. EPS from strain CAM025 was polyanionic and may sequester dissolved organics or bind cations such at trace metals. Therefore, the role of bacterial EPS in the Antarctic marine environment may have important ecological implications.

CAM025 was grown in broth culture at 0°C and the EPS produced was purified by ultrafiltration to investigate the metal binding ability of this high molecular weight, high viscosity polysaccharide. The preliminary study showed that the EPS had a high affinity for both cadmium and copper. These initial data provide insight into the ability of EPS such as these to chelate dissolved iron in the Antarctic marine environment. In the Southern Ocean iron limits primary productivity and the resulting draw-down of carbon dioxide, an important green house gas, from the atmosphere. Microbial EPS in suspended aggregates of marine snow may influence the availability of dissolved iron for primary production in Antarctic

waters. This study represents a first step in the understanding of the role a bacterial EPS in the Antarctic marine environment.

Deep-sea hydrothermal vent environments are characterized by high pressure, high temperature, heavy metals and novel microorganisms. The commercial value of microbial EPS from deep-sea vent habitats has been established recently. The microbial biodiversity of Antarctic ecosystems is relatively unexplored. Extreme environments offer unique microbial biodiversity that produce varied and promising EPS. The biotechnological potential of these biopolymers from Antarctic marine ecosystems remains largely untapped. Future research into possible commercial applications for EPS produced by Antarctic marine bacteria is warranted.

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